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American Journal of Veterinary Research

VOL. III OCTOBER, 1942 NO. 9

The Diagnosis of Actinomycosis and Actinobacillosis

M. S. SHAHAN, D.V.M., and C. L. DAVIS, D.V.M.

U. S. Bureau of Animal Industry
Washington and Denver

TECHNICALLY SPEAKING, actinomycosis means infection by any of the many species of organisms in the genus Actinomyces.[1] Clinically, the term is practically confined in the case of cattle to infections characterized by hard swellings of the tongue and other organs and bony enlargements of the jaw, as originally described by Bollinger,[2] although often other purulent infections attended by granulomatous processes are indiscriminately included. Harz, a botanist, suggested the name *Actinomyces bovis* for the fungus-like organism present in the numerous, opaque, yellowish granules (sulfur granules) that characterize the pus of actinomycosis.[3]

A disease resembling actinomycosis of cattle was encountered in man by Israel[4] in 1878-9, and in 1879 Ponfick[5] suggested that the two diseases were identical. In 1890, Bostroem[6] reported demonstration of aërobic, branching, filamentous organisms from bovine cases and in 1891 Wolff and Israel found morphologically similar, but anaërobic, organisms in human cases.[7] In 1905, Wright[8] made a detailed study of actinomycosis in man and cattle. The organisms to which he assigned etiologic significance were anaërobes like those originally described by Wolff and Israel. The aërobic organisms in the tissues described by Bostroem and others closely resembled those readily found in soil and on grasses, cereals, and other vegetable materials. Fragments of grass and awns of various plants were frequently observed in the lesions of actinomycosis, and it was assumed that the organisms from these foreign substances caused the disease. The evidence contradicting this view is well summarized by Topley and Wilson[9] and the British Medical Research Council.[10] The present general conception is that only anaërobic or microaërophilic organisms should be classed as *A. bovis*, and that these, which are probably strictly parasitic organisms, rather than the widely distributed saprophytic Actinomyces (spp), are the cause of true actinomycosis.

From the Pathological Division, U. S. Bureau of Animal Industry, Washington, D. C. (Shahan) and Branch Pathological Laboratory, Denver, Colo. (Davis).

[1]Bergey, D. H., Breed, R. S., Murray, E. G. D., Hitchens, A. P., and others: Bergey's Manual of Determinative Bacteriology, 5th ed., Williams and Wilkins, Baltimore, Md., (1938).

[2]Bollinger, O.: Ueber eine neue Pilzkrankeit beim Rinde. Zbl. med. Wiss., xv (1877), p. 481.

[3]Harz, C. O.: 1877-8, Actinomyces bovis, Eine neuer Schimmel in den Geweben des Rindes. Jahresb. d. Central—Thierärznei—Schule, München, in Deut. Zeitsch. f. Thiermed. u. vergl. Path., v (1879), pp. 125-140.

[4]Israel, J.: Neue Beobachtung auf dem Gebiete der Mykosen des Menschen. Virchow's Arch. path. Anat. Phys., lxxiv (1878), p. 15; *ibid.*: Neue Beiträge zu den mykotischen Erkrankungen des Menschen. *Ibid.*, lxxviii (1879), p. 421.

[5]Ponfick: Achter Congress der Deutschen Gesellschaft für Chirurgie zu Berlin. Berl. klin. Wschr., xvi (1879), p. 345.

[6]Bostroem, E.: Untersuchungen über die Aktinomykose des Menschen. Beit. path. Anat. allg. Path., ix (1890), p. 1.

[7]Wolff, M., and Israel, J.: Ueber Reincultur des Actinomyces und seine Uebertragbarkeit auf Thiere. Virchow's Arch. path. Anat. Phys., cxxvi (1891), p. 11.

[8]Wright, J. H.: The Biology of the Microörganisms of Actinomycosis. J. Med. Res., viii (1905), p. 349.

[9]Topley, W. W. C., and Wilson, G. S.: The Principles of Bacteriology, 2nd ed., Wm. Wood and Co., Baltimore, Md. (1936).

[10]British Medical Research Council: A System of Bacteriology in Relation to Medicine, H. M. Stationer's Office, London, viii (1931).

Actinomycosis has been diagnosed many times entirely on the basis of the presence of rayed formations of elongated, club-shaped, eosinophilic, hyaline-like structures, sometimes referred to as rosettes, in pus or sections. Lignières and Spitz[11] were the first to show that other organisms besides Actinomyces might be attended in the tissues by radiating club formations, or rosettes. The lesions found in Argentine cattle by Lignières and Spitz were reproducible by inoculation of a nonmotile, nonbranching, gram-negative, aërobic coccobacillus, which they called actinobacillus, now known as *Actinobacillus lignièresi*. The disease, actinobacillosis, has since been widely identified and studied. Higgins[12] in Canada; Griffith,[13] Bosworth,[14] and Davies and Torrance[15] in the British Isles; Gunst[16] in Holland; Bongert[17] in Germany; Hulphers[18] and Magnusson[19] in Sweden; and Thompson[20] and Vawter[21] in the United States have studied both actinobacillosis and actinomycosis and agree generally with Lignières and Spitz. Still, as stated by Vawter in 1933,[21] "The work of Lignières and Spitz has not received the attention it warrants." Distinctions between actinomycosis and actinobacillosis are not fully recognized and appreciated even today, with the consequence that there are wide discrepancies in clinical reports. Failure to distinguish one disease from the other has led to diversity of opinion as to the efficacy of iodine therapy. Sodium iodide administered intravenously, as originally recommended by Farquharson,[22] has been widely used. The treatment has been generally acclaimed as practically specific for actinobacillosis, but there is some question as to its efficacy in cases of actinomycosis, especially those with bone involvement.

In botryomycosis, preferably called staphylococcosis by some since it is now generally attributed to staphylococci rather than so-called Botryomyces, granules resembling those of actinomycosis are formed in the purulent contents of the granulomata.[9 19] Microscopically, these consist of gram-positive masses of micrococci enclosed in a zoögleal substance. About these masses, radiating hyaline-like clubs are sometimes found particularly in bovine udder lesions. Horses and swine, as well as cattle, may be affected.

In the study of coccidioidal granuloma in cattle, it has been observed by one of the writers (C. L. D.) that *Coccidioides immitis* may lead to the formation of rosettes in the tissues.[23] The rosettes when present in the purulent contents of coccidioidal granulomata may be seen in press preparations or in histologic sections (fig. 1). The presence of the spherules from which the clubs radiate permits differentiation from other suppurative processes with radiating clubs. It has been reported that hyaline clubs may be formed about dead masses of tubercle bacilli under experimental conditions and that they could occur in some cases of natural tuberculosis.

Abscesses due to *Corynebacterium pyogenes* are frequently found in the lymph nodes or elsewhere in cattle and other animals. These lesions may be mistaken for actinobacillosis or actinomycosis. In these cases, however, no rosettes have been ob-

[11]Lignières, J., and Spitz, G.: Actinobacillose. Rev. de la Sociedad Médica Argentina, x (1902), p. 5.

[12]Higgins, C. H.: Actinobacillosis. Proc. 41st Mtg. A.V.M.A., (1904), p. 131.

[13]Griffith, F.: On the Pathology of Bovine Actinomycosis. J. Hygiene, xv (1916), p. 195.

[14]Bosworth, T. J.: The Causal Organisms of Bovine Actinomycosis. J. Comp. Path. Therap., xxxvi (1923), p. 1.

[15]Davies, G. O., and Torrance, H. L.: Observations Regarding the Etiology of Actinomycosis in Cattle and Swine. J. Comp. Path. Therap., xliii (1930), p. 216.

[16]Gunst, J. A.: Over Verwekkers van Actinomycose bij het Rund. Tidschr. v. Diergeneesk., liv (1927), p. 497.

[17]Bongert, J.: Die Aetiologie der Aktinomykose bei Rindern und Schweinen. Zeitschr. f. Fleisch u. Milchhyg., xxxiv (1924), p. 251.

[18]Hulphers, G.: Fortsatta Undersökningar rörande Nötkreaturs Aktinomykosens Ätiologi. Svensk. Veterinärtidskrift, xxviii (1923), p. 273.

[19]Magnusson, H.: The Commonest Forms of Actinomycosis in Domestic Animals and their Etiology. Acta Path. et Microbiol. Scand., v (1928), p. 170.

[20]Thompson, L.: Actinobacillosis of Cattle in the United States. J. Infect. Dis., lii (1933), p. 223.

[21]Vawter, L. R.: A Study of Actinomycosis. Cornell Vet., xxiii (1933), p. 126.

[22]Farquharson, J.: Intravenous Use of Sodium Iodide in Actinomycosis. J.A.V.M.A., xci (1937), p. 551.

[23]Davis, C. L., and Stiles, G. W.: Actinobacillosis in Rams. J.A.V.M.A., xcv (1939), p. 754.

served, according to reports. Nor have rosettes been reported in other pyogenic infections not specifically mentioned here. The main confusion, or rather lack of specific recognition, is with respect to actinomycosis and actinobacillosis, the subjects of this paper.

In 1938, the writers made a study of 28 unselected cases of suspected actinomycosis and actinobacillosis in slaughtered cattle in an attempt to correlate or distinguish the macroscopic, cultural, and histopathological findings in the two conditions. Lesions of soft tissues and bone were included in these cases. In each instance, the lesions were examined grossly; histologic sections were prepared; the pus was examined in press preparations, and the size and morphology of the rosettes were noted; the material was ground in sterile sand and cultured; and a Gram stain was made of both smears and sections as an aid in determining the character of the organisms involved in each case. The pathological and cultural examinations were conducted independently, and after completion of the study it was found that the two methods of diagnosis showed a close correlation. The distribution of the lesions and the diagnosis of the cases in this group are shown in table I. The findings were essentially the same as those reported by other investigators referred to above. The results indicated that a differential diagnosis could be satisfactorily made in the majority of cases without resorting to cultural methods.

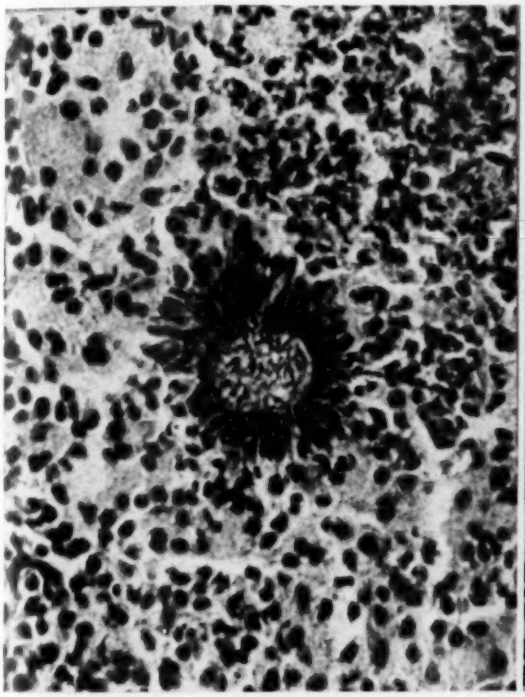

Fig. 1—Section of bovine coccidioidal granuloma in lung, showing a spherule surrounded by a well defined ray of clubs. Stained by hematoxylin-eosin. x500.

Fig. 2—Section of actinomycosis in bone, stained by hematoxylin-eosin, showing a granule composed of a central mass of organisms surrounded by a fringe of radiating clubs. x450.

Fifty additional cases in different species of animals in which soft tissues only were involved were submitted to the Denver laboratory for diagnosis. Of the 50 cases, actinomycosis of soft tissues other than lymph nodes was diagnosed in 4 animals with involvement of reticulum, lung, and mucosa of the frontal sinus (see table II). In the actinomycotic cases of this group there were no associated bone lesions. Lesions in the lymph nodes only, or in com-

TABLE I.—Summary of cultural and pathological observations.

LOCATION OF LESIONS	NO. OF SPECIMENS	ACTINOBACILLOSIS	ACTINOMYCOSIS
Cervical lymph nodes	18	18	..
Cervical lymph nodes and lung	1	1	..
Submaxillary salivary gland	1	..	1
Tongue	1	1	..
Lung only	1	1	..
Jaw (mandible)	5	..	5
Jaw and lymph nodes*	1	1	1
Totals	28	22	7

*This case presented the two types of infection in the same animal. The jaw lesion was actinomycosis and the lesions in the submaxillary, postpharyngeal and prefemoral lymph nodes were actinobacillosis.

mycosis of the mandible and actinobacillosis of the submaxillary, postpharyngeal, and prefemoral lymph nodes, but to date both conditions within the same lesion have not been observed by the writers.

LABORATORY FINDINGS

Grossly, the purulent contents of the lesions of actinomycosis and actinobacillosis are similar. The pus is viscid and greenish-yellow. Actinomycotic pus contains sulfur granules that are visible to the unaided eye, while in actinobacillosis, the granules are in the form of thin, whitish

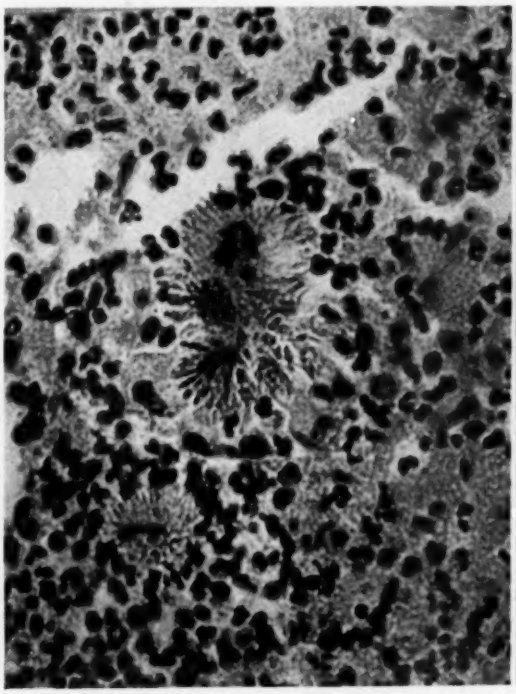

Fig. 3—Section of actinobacillosis in a lymph node, showing two small rosettes with pointed, clubbed ends. Stained by hematoxylin-eosin. x450.

Fig. 4—A gram stain of a section of actinomycotic lung in a hog, with diphtheroid and filamentous forms predominating. A faint fringe of pale unstained clubs surrounds the organisms. x2000.

bination with other lesions, are generally actinobacillosis. However, actinobacillosis without lymph node involvement was also observed in lung, liver, peritoneum, tongue, buccal membrane, muscle, and subcutaneous tissues. Occasionally, both diseases may be found in the same animal; bacteriological and pathological study of one such case, shown in table I, revealed actino-

flakes, usually visible only with the aid of a hand lens or microscope. Press preparations of the pus in both diseases reveal rosettes, but in our experience these are usually larger in actinomycosis, especially in the bone lesions where the central mass of organisms is fringed by a narrow peripheral zone of radiating clubs (fig. 2). Smears stained by Gram's method show

large numbers of gram-positive, coccoid, bacillary, and filamentous forms of varying lengths with occasional branching forms in actinomycosis, while in actinobacillosis no gram-positive organisms are seen.

The organisms differ materially in their cultural and morphological characteristics. *A. bovis* grows only under conditions of reduced oxygen tension and produces fine, slightly raised colonies on solid media after 48 to 96 hours of incubation. The organism is usually rod-shaped or filamentous, but occasionally coccoid forms, some resembling streptococci, are seen. Some difficulty may be experienced in recovering the organism in pure culture from bone lesions. Other organisms, such as *Corynebacterium pyogenes* and staphylococci, are sometimes encountered even in closed lesions. *Actinobacillus lignièresi* grows under aërobic conditions and produces small, bluish-grey colonies within 24 hours on serum agar which somewhat resemble the colonies of *Escherichia coli*. The organism is a short, oval, nonmotile, gram-negative, coccobacillus which produces acid without gas in dextrose, sucrose, galactose, maltose, levulose, and mannite. Other sugars, such as lactose, raffinose, glycerol, and sometimes xylose may be acidified after extended incubation. Litmus milk is slightly acidified, but not coagulated. Dulcite, rhamnose, salicin, and inulin are not fermented. The organism is agglutinated by immune serum.

Growths of either *A. bovis* or *Actinobacillus lignièresi* are facilitated by grinding the pus in a mortar which breaks down the clubbed masses about the organisms. The more compact rosettes of actinobacillosis enclose relatively few organisms and only a few colonies may develop unless the medium is very heavily seeded with well-triturated inoculum. The use of serum in agar or other medium accelerates the growth of both organisms, as well as others which require some such material for growth, such as *Cor. pyogenes*.

Histologically, the two diseases are gran-

TABLE II.—Summary of Cases Diagnosed Pathologically.

Species	Location of Lesions	No. of Cases**	Diagnosis: Actinobacillosis	Diagnosis: Actinomycosis
Cattle	Cervical lymph nodes only	20	20	..
"	Cervical lymph nodes and tongue	3	3*	..
"	Cervical lymph nodes, lung, peritoneum	1	1	..
"	Cervical lymph nodes and larynx	1	1	..
"	Cervical and mediastinal lymph nodes	1	1*	..
"	Tongue only	3	3*	..
"	Lung only	2	1	1
"	Lung and cervical bronchial lymph nodes	1	1	..
"	Lung, liver and mediastinal lymph node	2	2	..
"	Mediastinal lymph node only	1	1	..
"	Skeletal lymph nodes only	1	1*	..
"	Peritoneum only	3	3	..
"	Peritoneum, pleura, skeletal lymph nodes	1	1	..
"	Liver only	1	1	..
"	Buccal membrane and masseter muscle	1	1*	..
"	Reticulum only	1	..	1
"	Frontal sinus only (mucosa)	1	..	1
"	Subcutaneous only (head)	1	1	..
Sheep	Face and cervical lymph nodes	2	2*	..
"	Face and lung	1	1*	..
"	Lung, liver, spleen and pleura	1	1*	..
Hog	Lung only	1	..	1
		50	46	4

*Indicates positive cultures.

**In addition to the cases listed, 7 others were examined, but, because of failure for various reasons to reach a definite diagnosis, these are not included in the summary of cases. The site and nature of the lesions in these cases were as follows:

3 bovine udders—rosettes with gram-positive micrococci; cultures not obtained; tentatively diagnosed as staphylococcosis (botryomycosis).

1 bovine pancreas—rosettes in the presence of necrosis of the organ; cultures not obtained.

2 cases of multiple lesions in swine (lung, liver, spleen, mammae and lymph nodes in 1 case suggestive of actinomycosis; liver, spleen, kidney, stomach wall and lymph nodes in 1 case suggestive of actinobacillosis).

1 salivary gland from a mule, pathologically suggestive of actinomycosis; not cultured.

Fig. 5—A gram stain of a section of actinomycotic bone, showing myriads of coccoid and diphtheroid organisms surrounded by a faint halo of weakly stained clubs. x1600.

ulomatous in character, starting as suppurative processes which are eventually replaced by connective tissue cells and the formation of granulation tissue. The tissue and cellular reactions are essentially the same and afford no means of exact differentiation.

There is, however, a difference in the size of the rosettes in the two conditions. In the sections of actinomycotic lesions, the club formations are irregularly shaped and variable in size, and as a general rule are considerably larger than the uniformly small rosettes present in actinobacillosis (fig. 3). Individual clubs, which have apparently broken away from the central rosettes, may be seen in either disease. The rosettes in actinobacillosis, as a rule, have well-defined and perhaps more pointed clubbed ends than those found in actinomycotic lesions.

Giant cells, sometimes containing frag-

ments of rosettes, are usually present as a part of the cellular reaction in both diseases and are commonly seen surrounding the rosettes in the granulomatous areas of the lesions. As the lesions become older, many of the rosettes undergo calcareous changes. It is in this stage that actinobacillosis of the cervical lymph nodes is more likely to be confused with tuberculosis or coccidioidal granuloma. In fact, 9 of the 20 cases of cervical actinobacillosis, listed in table II, were diagnosed grossly as tuberculosis, and 4 of these 9 cases were found in tuberculin reactors. Diagnosis was established by the absence of acid-fast organisms in smears or sections and by the presence of gram-negative organisms within the radiating clubs. Davies and Torrance[15] have reported 2 cases of coexistent actinobacillosis and tuberculosis in cattle.

In some cases of long standing, particularly in lesions of the head, there is extensive replacement of the granulomatous tissue by connective tissue, forming a tumor-like mass which may be mistaken for a fibroma. Cut and stained sections made from several areas of such lesions

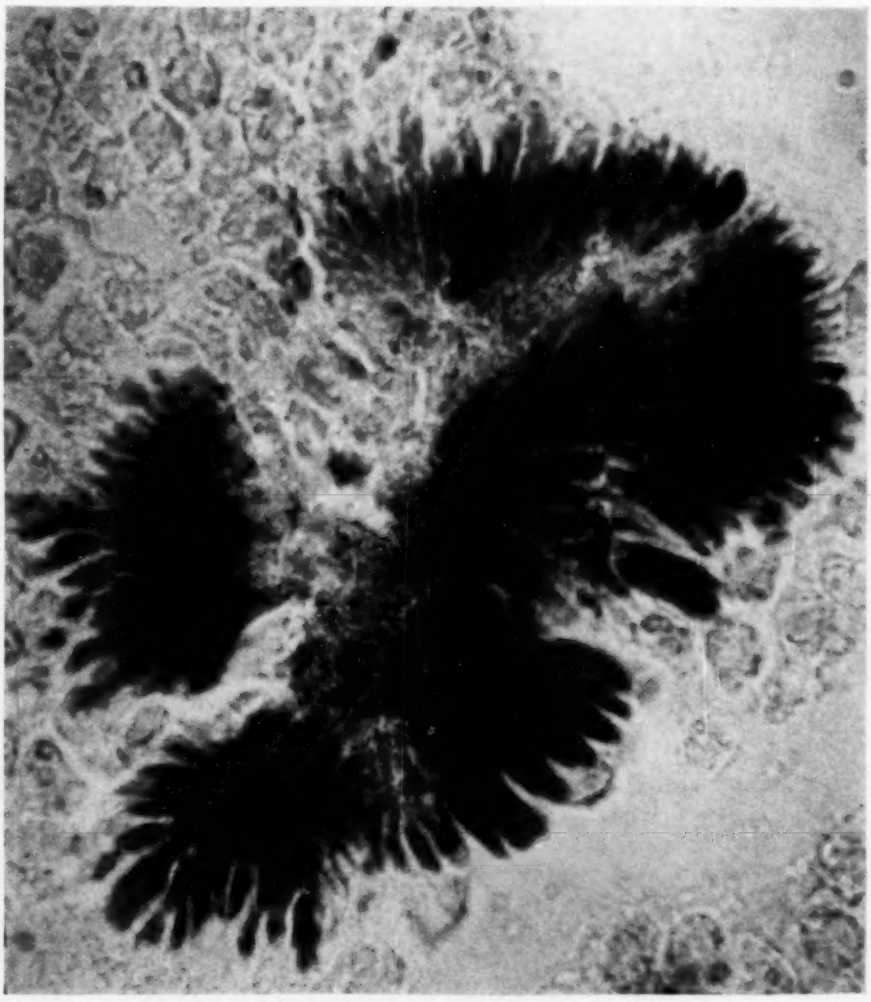

Fig. 6—A section of actinobacillosis in a lymph node stained by eosin and methylene blue, showing several clumps of short bacillary forms. Note the pointed club forms in the eosin-stained rosette. x1600.

often will reveal a few remaining rosettes in the scar tissue.

In resorting to histologic means of diagnosis, it is necessary to stain the tissue for the presence of organisms. In actinomycosis, the organisms are gram-positive and in section appear as coccoid, diphtheroid, or filamentous forms within the rosettes (fig. 4). In some instances coccoid and diptheroid forms are predominant (fig. 5). In actinobacillosis, the organisms are gram-negative rods and are readily demonstrable in comparatively limited numbers within the rosettes, either by the Giemsa or eosin-methylene-blue stain (fig. 6). Occasionally the clubbed ends of some rosettes in actinobacillosis and actinomycosis retain the Gram stain and may be mistaken for gram-positive organisms.

At the present time, so far as the writers are aware, *Actinobacillus lignièresi* is the only gram-negative organism that is associated in the tissues with rayed clubs resembling those of actinomycosis. This peculiarity alone may serve to identify actinobacillosis. However, the situation with respect to gram-positive organisms and actinomycosis is somewhat different. In the case of bone lesions with the clinical appearance of actinomycosis, diagnosis based on demonstration of gram-positive, diphtheroid or filamentous organisms within the rayed clubs is acceptable in most cases, according to present knowledge. On the other hand, in the case of lesions with gram-positive organisms in other tissues, such as lung or udder, it is preferable to prepare cultures. It is known that the zoögleal masses found in botryomycosis (staphylococcosis) may be surrounded by rayed clubs, as may *Coccidioides immitis*. The possible existence of still other gram-positive organisms, such as *A. farcinicus* and *A. asteroides*, or the gram-negative *Actinobacillus actinoides* and *Actinobacillus actinomycetem-comitans* (Bergey's Manual), should not be overlooked. Classification of the entire Actinomycetales group is in a very unsettled state,[24] and present indications are that many, rather than few, of the Actinomyces are pathogenic for the lower animals.[25]

As concerns the formation of hyaline clubs, present information considerably detracts from the conception of these formations as part of the etiologic organism. Diverse species of organisms appear to induce formation of these structures. They seem to be a product of tissue reaction, rather than a part of the organisms themselves.

SUMMARY

In 78 animals having lesions suspected as being either actinomycosis or actinobacillosis, 68 cases were diagnosed as actinobacillosis and 11 as actinomycosis. One animal, as proved by cultures, showed both infections. The cases showing lymph node involvement, alone or in combination with other lesions, were all found to be actinobacillosis. Of the 11 animals showing actinomycosis, 6 were bone cases and 5 had lesions of various organs not associated with bone or lymph node involvement.

While the two diseases are commonly considered in the same category from a clinical point of view, technically they are distinct entities. Although the causative organisms in both diseases are capable of producing rosettes, they differ materially in their cultural and morphological characteristics. *Actinomyces bovis* is anaërobic or micro-aërophilic and is gram-positive, while *Actinobacillus lignièresi* is aërobic and gram-negative. Cultural methods, when possible, are most desirable to differentiate these diseases from other suppurative processes. Our studies indicate, however, that a practical differential diagnosis of actinomycosis and actinobacillosis can be made satisfactorily in many, if not most, instances without resorting to cultures. The rosettes in actinomycosis are generally more variable in shape and size and are usually larger than the uniformly small rosettes seen in actinobacillosis.

Histologically, the two diseases show

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A Study of the Administration of Sulfanilamide to the Normal Dairy Cow

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WITHIN THE LAST few years, sulfanilamide has been given more attention than perhaps any other drug in the treatment of bovine mastitis.[1, 2, 9, 14, 15] Only a few clinical studies of the administration of sulfanilamide to the normal dairy cow have appeared in the literature. Perhaps the first report dealing with the elimination of sulfanilamide in the milk was made by Schlotthauer in 1938.[16] Later, Gildow, *et al.*,[5] made one of the most intensive studies of the absorption of sulfanilamide by determining its concentration in the blood and milk as affected by different levels of dosage.

Only a study of the concentration of free sulfanilamide in the blood and milk has appeared in the literature. Marshall,[11] however, has shown that various animals may conjugate sulfanilamide as the acetyl derivative. The acetylated compound in most instances has been shown to be inactive. Hence, it was felt that the determination of conjugated sulfanilamide in the blood and milk would show a double significance. It would give a more accurate picture of the fate of ingested sulfanilamide and, furthermore, might explain the inefficiency of sulfanilamide in many cases of mastitis.

In view of the well-known toxic effects of sulfanilamide on the bone marrow of human subjects,[3, 7, 8] it seemed obvious that a study of the hemoglobin and the cellular constituents of the blood of dairy cows undergoing sulfanilamide administration would provide an index of the toxicity of the drug.

The present investigation deals with the effects of different doses of sulfanilamide on the normal dairy cow. This study was approached from two viewpoints: (1) a study of the free and total sulfanilamide content of the blood and milk, and (2) its effect on the blood picture; namely, on the erythrocyte, leucocyte, and differential counts and the hemoglobin content. The term total sulfanilamide includes free sulfanilamide as well as that present in the conjugated form. During the course of the investigation, a careful record was made of the effect of sulfanilamide on the daily milk production.

EXPERIMENTS

In this study, four Holstein-Friesian cows of known weight, free from mastitis and normal in all other respects, were chosen from the dairy herd of the Department of Dairy Husbandry, Pennsylvania State College.

Blood samples were drawn from the jugular vein daily after the 2:00 p.m. milking period. It has been found that bleeding just before milking interferes with milk production. The cows were milked each day at 6:00 a.m., 2:00 p.m., and 10:00 p.m. One one-hundredth aliquots were taken at each milking period and pooled, and a sample was taken from the pooled aliquots for analysis of free and total sulfanilamide.

During the first control period, neither milk nor blood was analyzed for sulfanilamide, since previous tests had indicated that normal blood and milk give no color with the reagents used in the determination of sulfanilamide. During the first experimental period, the sulfanilamide

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similar tissue and cellular reactions and can best be differentiated by the presence or absence of gram-positive organisms within the rosettes. Clinically and *post mortem*, these diseases may be confused with staphylococcosis (botryomycosis), tuberculosis, coccidioidal granuloma, and lesions produced by *Corynebacterium pyogenes* or other pyogenic organisms. Therefore, laboratory examination is often necessary for diagnosis which is especially important from the standpoints of prognosis and treatment.

was administered in half-ounce gelatin capsules with a balling gun in 2 equal doses at 12-hour intervals, at 9:00 a.m. and at 9:00 p.m. Since the second level of dosage necessitated the use of a large number of capsules, it was found advisable, during the second experimental period, to administer the sulfanilamide by drenching. The administration of sulfanilamide was discontinued during the fourth period of the investigation, designated as the final control period. Milk samples were taken daily until both free and conjugated sulfanilamide could no longer be detected in them. Blood samples also were drawn daily until the hemoglobin values and the cell counts had returned to normal. At the conclusion of the investigation, the animals were weighed to determine the loss in weight following the use of sulfanilamide.

Free and total sulfanilamide were determined in a laked blood filtrate, according to the method of Marshall.[10] With a slight modification, the method was adapted for the determination of the above in milk; water was used as the diluent instead of saponin solution. In all other respects, the procedure was identical for both blood and milk. The procedure for determining both free and total sulfanilamide is the same except that in the latter, an aliquot of the blood or milk filtrate is first hydrolyzed. The results for both are given in milligrams per 100 cc. of sulfanilamide.

Erythrocyte and leucocyte counts were made in duplicate in the usual manner. Hemoglobin was determined by the acid hematin method of Newcomer,[13] using a standard color plate. All colorimetric readings were made in daylight. For the differential count, Wright's stain was employed, a phosphate buffer of pH 7.0 being used as the diluent in place of distilled water. For each determination, 200 cells were counted, 100 from each of two duplicate slides.

A summary of the plan of the investigation is given in table I. It was necessary to modify the original plan to prevent overdosage, as explained in the following section.

TABLE I.—Showing the Plan of the Investigation

PERIOD	DATE	DOSAGE PER DAY PER 100 LB. BODY WEIGHT	DETERMINATIONS MADE ON BLOOD	DETERMINATIONS MADE ON MILK
Initial control	July 10-21, 1939	None	Erythrocyte count Leucocyte count Differential count Hemoglobin	None
First experimental	July 24-31, 1939	5 Gm.	Erythrocyte count Leucocyte count Differential count Hemoglobin Free sulfanilamide Total sulfanilamide	Free sulfanilamide Total sulfanilamide
Second experimental	July 31-Aug. 7, 1939	10 Gm.		
Final control	Aug. 7-18, 1939	None		

PRESENTATION OF DATA

Four cows, 184, 186, 204, and 239, were chosen for this investigation. Since the results obtained on the different cows were in reasonable agreement, only the data for two animals, cows 184 and 204, are presented.

During the course of this investigation, it became evident that the original plan of the investigation as outlined in table I could not be followed, since the animals reacted unfavorably to the drug. In every case, the cows went off feed at some period during the study. The administration of sulfanilamide was then discontinued for a period and instituted again as indicated on the graphs in fig. 1. Symptoms which necessitated discontinuance of the drug were a slightly inflamed udder, diarrhea, and a general unfavorable systemic appearance. The cows did not ruminate, were listless, and seemed to be in a state of general malaise. It is possible that the diarrhea was due in part to the ration, since freshly cut soybean forage and Sudan grass were fed during the period when the

diarrhea was noticed. Refusal of the feed for more than one day was considered sufficient cause to discontinue the administration of sulfanilamide.

A.—Concentration of Total, Conjugated, and Free Sulfanilamide in the Blood and Milk.—The concentration of total, conjugated, and free sulfanilamide in the blood and milk following administration of the drug is presented in fig. 1. The concentration of the conjugated or acetylated sulfanilamide was calculated by taking the difference between the values representing total and free sulfanilamide. The lower level of dosage, 5 Gm. per 100 pounds of body weight daily, is referred to as the first dosage, and the higher level is called the second dosage. The legend for the curves in parts *A* and *C* of fig. 1 also refers to parts *B* and *D*, for the same animals respectively.

On the low level of dosage, the maximum concentration of total sulfanilamide in the blood of cow 184 was found to be 6.5 mg. per 100 cc., whereas in the milk of the same animal, a concentration of 12.7 mg. per 100 cc. was reached. At first glance, it seems strange that the concentration of total sulfanilamide in the milk should exceed that in the blood. An examination of the partition of total sulfanilamide, however, shows that about twice as much of the conjugated form is present in the milk as in the blood. In the blood, a maximum concentration of 2.4 mg. per 100 cc. of conjugated sulfanilamide was found, representing 37 per cent of the total sulfanilamide, while in the milk, 8.8 mg. per 100 cc. were conjugated, representing 69 per cent of the total sulfanilamide. On the same level of dosage, the free sulfanilamide distributed itself approximately equally in the blood and milk, 4.7 mg. per 100 cc. being the maximum found in the blood, and 4.2 mg. per 100 cc., the maximum found in the milk.

On the high level of dosage, however, it was surprising to note that sulfanilamide was present in the blood almost wholly in the free form. A maximum concentration of free sulfanilamide of 17.5 mg. per 100 cc. was reached in the blood of cow 184. Despite the fact that little conjugated sulfanilamide could be found in the blood on the high level of dosage, a large amount was present in the milk. A maximum concentration of total sulfanilamide in the milk of 27.4 mg. per 100 cc. was reached. For the same day, the milk showed a concentration of conjugated sulfanilamide of 15.4 mg. per 100 cc., representing 56 per cent of the total sulfanilamide. The free sulfanilamide was found to reach a concentration of 12.0 mg. per 100 cc.

It will be noted that, in general, the concentration of free sulfanilamide in the milk did not exceed that in the blood on either level of dosage. From this it would appear that the passage of the unconjugated compound from the blood into the milk could be accounted for on the basis of diffusion. However, when one examines the data on conjugated sulfanilamide, it is more difficult to explain how the concentration in the milk is higher on both levels of dosage than that in the blood. Obviously, diffusion is not the complete answer. It is still more difficult to explain why on the high level of dosage there is so little conjugated sulfanilamide in the blood, when the concentration in the milk is high.

It should be pointed out that in this study no attempt has been made to determine the nature of the conjugated compound. Marshall[11] has found that, in animals where conjugation occurs, the conjugated compound is the acetyl derivative, and we have assumed that this is the case in this study. However, it would seem possible that many compounds other than acetic acid in the blood and milk might combine with sulfanilamide. Marshall[11] has also stated that conjugation takes place mainly in the liver. The results obtained in this study might be interpreted by assuming that the mammary gland also has the ability to conjugate sulfanilamide. It would appear possible that the conjugated compound in the milk may not be the same as that in the blood. Since milk has quite a different composition from that of blood, it is possible that, in the milk, sulfanila-

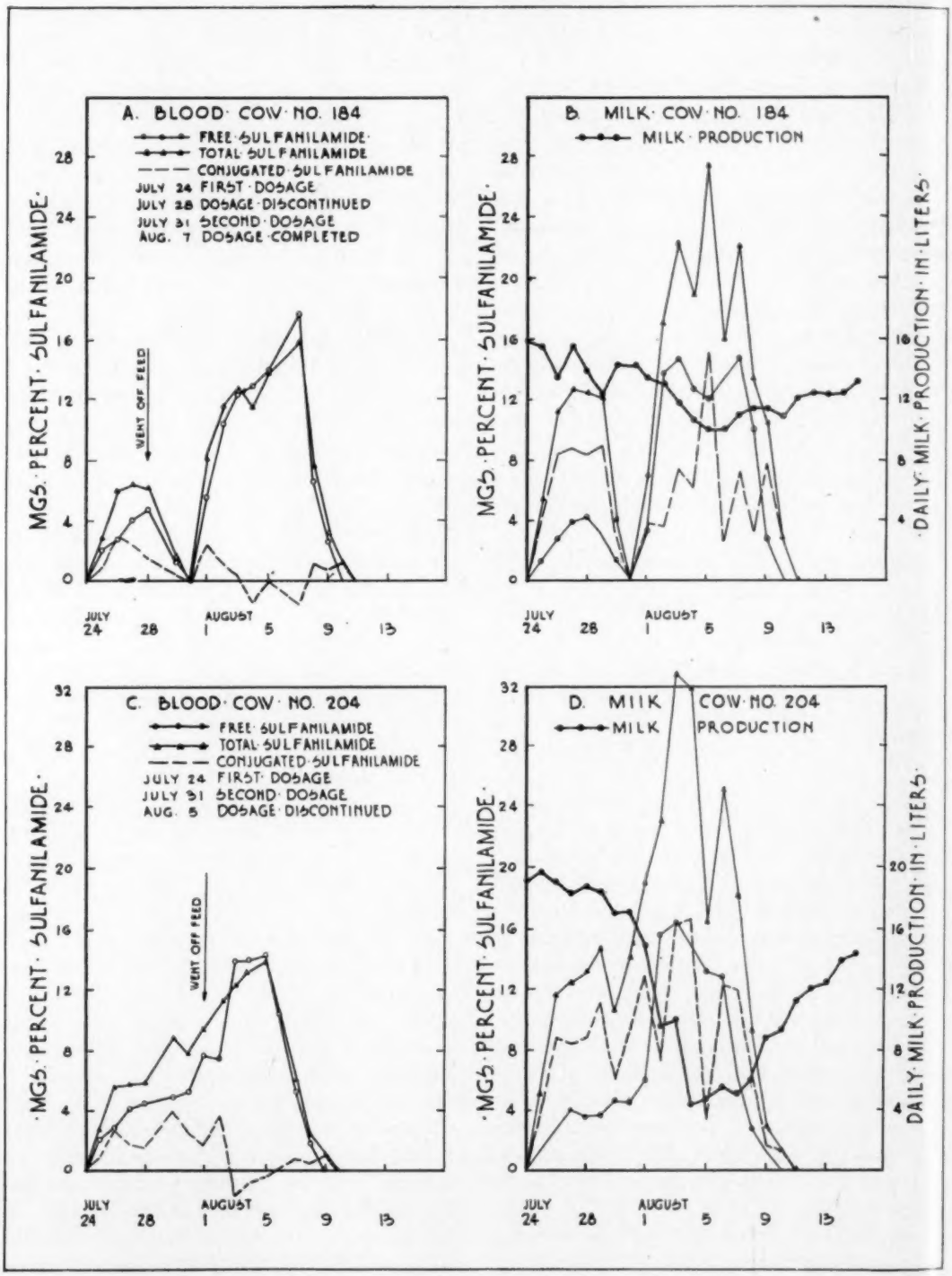

Fig. 1—The effect of sulfanilamide administration on the concentration of total, conjugated and free sulfanilamide in the blood and milk.

mide is conjugated with some constituent not found in the blood. An example of such a possibility would be the formation of a glucoside complex with lactose. If such compounds were formed, it would not be difficult to explain the high concentration of conjugated sulfanilamide in the milk.

The low concentration of conjugated sulfanilamide in the blood on the high level of dosage might be accounted for in two ways: (1) the concentration of free sulfanilamide in the blood on the high level of dosage may be sufficient to injure the liver tissue and thus interfere with its conjugating function, and (2) it is possible that with long continued administration, the compound in the blood which normally conjugates with it may be depleted. In that case it would be expected that no more conjugation would take place.

It would be interesting to attempt to isolate the conjugated derivatives from blood and milk and determine their nature. With this information, it should be possible to give a more satisfactory explanation of the high concentration of conjugated sulfanilamide in the milk.

With the advent of sulfanilamide, it was hoped that it would prove to be a valuable therapeutic agent in the treatment of mastitis. The high concentrations of total sulfanilamide found in the milk in this study would appear to favor the use and efficacy of the drug in this condition. It should be noted, however, that the greater proportion of sulfanilamide in the milk exists in the conjugated form, which has been shown to be therapeutically inactive. It has also been shown that free sulfanilamide tends to distribute itself equally in the blood and milk, in levels therapeutically effective in many cases of streptococcic infections. Since many cases of mastitis do not respond to sulfanilamide therapy, and since the concentration of the drug in both the blood and milk is sufficient for other therapeutic uses, it would appear that the organisms responsible for mastitis are not particularly sensitive to sulfanilamide.

Three days were required for the free sulfanilamide to disappear from the blood and milk of animal 184 following the last dose. An additional day was required for the conjugated compound to disappear completely from the blood and milk.

The daily milk production during the experimental and the final control periods is also presented in fig. 1*B* and 1*D*, to show the correlation between the milk flow and the concentration of sulfanilamide in the milk. As is particularly well illustrated in fig. 1*D*, the milk production varied inversely with the concentration of sulfanilamide in the milk. The milk flow returned to normal slowly, a maximum recovery of 83 per cent being observed in cow 184, 11 days after the final dose.

Similar results have been obtained for cow 204, except for the reduction in milk flow. A maximum reduction of 78 per cent was found in this animal, as compared with a 38 per cent reduction for animal 184. It should be remembered, however, that the same plan of dosage was not followed for both animals, as indicated in fig. 1*A* and 1*C*.

B.—Cellular Constituents of the Blood.—The effect of the administration of sulfanilamide on the cellular constituents of the blood, namely, the red and white cell count and the differential count, is presented in fig. 2. The hemoglobin value is also presented here to show its correlation with the erythrocyte count. To conserve space, only the data for cow 184 are presented. Normal values were determined for a period of 10 days preceding the experimental periods. The average value for the hemoglobin content and the cellular constituents of the blood is indicated in the graphs in fig. 2 by the straight lines designated "average normal." The administration of sulfanilamide is indicated by the arrows under the lymphocyte curve in the bottom graph of fig. 2 and applies to all the curves in the same figure. Since only the neutrophile and lymphocyte counts of the differential counts showed any significant variation from the normal, they alone are presented in fig. 2.

During the initial control period, an average hemoglobin value of 9.80 Gm. per 100 cc. of blood was obtained. On the low

level of dosage, a slight increase in the hemoglobin was produced. On the high level of dosage, however, a decreasing trend was noted which reached a minimum of 7.89 Gm. per 100 cc., representing a decrease of 19 per cent from the normal value for animal 184.

A normal erythrocyte count of 6,700,000 per cubic millimeter of blood was obtained. During the experimental periods, the erythrocyte response closely paralleled the hemoglobin values as indicated in fig. 2.

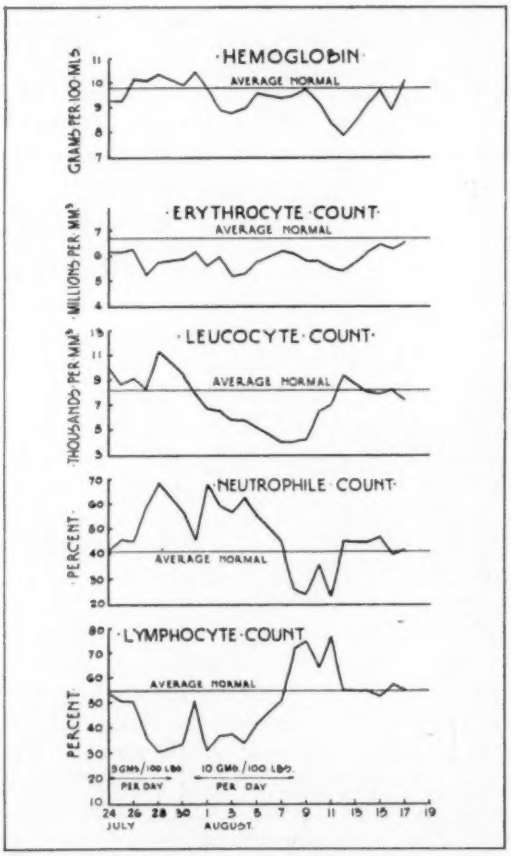

Fig. 2—The effect of sulfanilamide administration on the hemoglobin values and the blood cell counts.

The lowest value noted was 5,220,000 cells per cubic millimeter, representing a reduction of 22 per cent from the normal.

The normal leucocyte count for cow 184 was found to be 8,200. On the low level of dosage, a mild leucocytosis was produced. When the high level of dosage was instituted, there was a marked reduction in the leucocyte count. The response was rapid, a definite leucopenia being produced which reached a minimum of 3,960 per cubic millimeter of blood, representing a reduction of 52 per cent from the normal. After the dosage was discontinued, the leucocyte count rapidly returned to normal.

The normal differential count presented in the following table is the average of nine counts of 200 cells each made on cow 184 during the control period, representing an average based on 1,800 cells. No distinction was made between small and large lymphocytes, and the monocytes also included the so-called transitional cells.

TABLE II.—Showing Differential Leucocyte Count During the Initial Control Period

	PER CENT	TOTAL
Neutrophiles	41	3,362
Lymphocytes	55	4,510
Monocytes	1	82
Eosinophiles	2	164
Basophiles	0	0
Divided lymphocytes	1	82

The only significant change due to the administration of sulfanilamide was found in the proportion of neutrophiles and lymphocytes. There was a definite increase in the number of neutrophiles, which reached a maximum value of 69 per cent. As was to be expected, the lymphocyte count varied inversely with the neutrophile count. A definite lymphopenia was produced, reaching a minimum value of 31 per cent.

During the high level of dosage, atypical and degenerated leucocytes appeared in the blood. These include monocytes with a lobulated nucleus, large atypical monocytes, and lymphocytes in various stages of disintegration. The significance of these cells is not known. The blood studies were continued after sulfanilamide administration was discontinued until approximately normal results were obtained, at which point the investigation was concluded.

The normal values for cellular counts and hemoglobin presented in this paper are in close agreement with those reported in the literature.[4, 6, 12]

The animals were weighed at the conclusion of the study and showed an average

loss in weight of 8.6 per cent, as represented in table III.

TABLE III.—Loss of Weight Following Ingestion of Sulfanilamide

Cow No.	Wt. before July 20 lb.	Wt. after Aug. 7 lb.	Wt. Loss lb.	Wt. Loss %
184	1,240	1,150	90	7.3
186	1,265	1,200	65	5.1
204	1,250	1,090	160	12.8
239	1,100	1,000	100	9.1

SUMMARY

Four normal Holstein-Friesian cows were given sulfanilamide on two levels of dosage, 5 and 10 Gm. per 100 pounds of body weight per day. A study was made of the concentration of free and conjugated sulfanilamide in the blood and milk. In addition, a study was made of the hemoglobin values and the blood picture, namely, the erythrocyte, leucocyte, and differential counts.

On the low level of dosage, 69 per cent of the total sulfanilamide found in the milk was present as the conjugated compound, whereas, 37 per cent was present in the blood as the conjugated derivative. On the high level of dosage, 56 per cent of the total sulfanilamide was conjugated in the milk, whereas, little conjugated sulfanilamide could be detected in the blood on the same level of dosage. Free sulfanilamide distributed itself approximately equally in the blood and milk on both levels of dosage.

A slight anemia was produced, with a maximum reduction in the erythrocyte count and in the hemoglobin value of about 20 per cent. On the low level of dosage, a slight leucocytosis was observed, while on the high level of dosage, a marked leucopenia was produced, representing a decrease in leucocytes of 60 per cent. A decided increase in percentage of neutrophiles was noted. In addition, atypical and degenerated leucocytes were found in the blood during the height of sulfanilamide administration.

The milk production was found to vary inversely with the excretion of sulfanilamide in the milk. The milk flow was reduced by as much as 82 per cent.

An average decrease in weight of 8.6 per cent resulted from the ingestion of sulfanilamide.

Individual differences in tolerance towards sulfanilamide were noted in the cows throughout the course of this study.

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Studies on the Production of Specific Antibodies Against the Agent of Fowl Leucosis

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In 1935, Uhl, Englebreth-Holm, and Rothmeyer[1] were able to inhibit the activity of the fowl leucosis agent by sera from ducks previously injected with blood from chickens affected with fowl leucosis.

The experiments here reported were made in an attempt to study further the behavior of the causative agent of the fowl leucosis complex and the interrelation of the various types of this complex. The experimental data reported have been accumulated over a three-year period and consequently not all of the experiments conducted are reported here. The charts and reported experiments are confined to those appearing to have experimental significance. Reference to literature on the fowl leucosis complex is omitted, but can be found in articles published previously.

One-year-old ducks were used in these experiments as they are generally regarded as nonsusceptible to the fowl leucosis complex. Adult turkeys were also used because of the ease in securing quite large quantities of blood or serum. While a few cases of fowl leucosis have been reported in turkeys, the occurrence is so rare that one must regard that species as only slightly if at all susceptible. In all, 12 ducks and 12 turkeys were employed.

The ducks and turkeys were immunized by means of 15 intramuscular injections of 4 cc. each during the course of 30 days with blood from chickens suffering from myeloid leucosis. After the last injection, the immunized birds were bled at 10-day intervals. In order to maintain the possible antibody concentration, injections of 4 cc. of leucosis chicken blood were made every 14 days. Routine differential blood smears were made daily on all injected birds. There was no change in the blood picture at any time.

The immunized birds were bled and the blood allowed to coagulate at room temperature. The clot was then broken, the samples centrifuged, and the serum collected. The serum was heated to 55 C. for 45 minutes and stored at —10 C. for subsequent use. Serum has been held for 10 months without any apparent change.

Tests for possible inhibitor content were as follows: Twenty parts of duck or turkey serum, either individual or pooled samples, from immunized birds were mixed with 1 part of oxalated blood from chickens suffering from myeloid leucosis. For controls, 20 parts of physiological saline were mixed with 1 part of the same leucosis material. The mixtures were placed in an incubator at 37 C. for 2 hours so that any binding of immune serum with affected material might take place.

The infective agent and serum, or infective agent and physiological saline were injected intracardially into 6-week-old susceptible chicks secured from our own breeding flock. The inoculated groups were placed in isolated colony houses equipped with screen porches. Suitable uninoculated controls for all inoculated groups were established from birds that were full brothers or sisters of the inoculated groups.

From the results of experiment I, immune duck serum neutralized the infective agent of the myeloid type of fowl leucosis in oxalated chicken blood from fowl leucosis chickens, the positive birds in the inoculated controls being above 80 per cent. The uninoculated controls remained free from the disease during the 10-month period. There

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EXPERIMENT I

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Oxalated leucosis chicken blood..... plus 4 cc. Immune serum (duck) (Individual sample)	20 5 weeks old	0.2 cc. Intracardially	3	10 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Oxalated leucosis chicken blood..... plus 4 cc. Physiological saline solution.....	20 5 weeks old	0.2 cc. Intracardially	17	10 months

EXPERIMENT II

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Oxalated leucosis chicken blood..... plus 4 cc. Immune serum (turkey) (Pooled sample)..	20 5 weeks old	0.2 cc. Intracardially	4	10 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Oxalated leucosis chicken blood..... plus 4 cc. Physiological saline solution.....	20 5 weeks old	0.2 cc. Intracardially	14	10 months

EXPERIMENT III

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Centrifuged plasma leucosis chicken blood plus 4 cc. Immune serum (duck) (Individual sample)	20 6 weeks old	0.2 cc. Intracardially	1	10 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Centrifuged plasma leucosis chicken blood. plus 4 cc. Physiological saline solution.....	20 6 weeks old	0.2 cc. Intracardially	16	10 months

seemed to be no comparable difference between immune turkey serum and duck serum as shown in experiment II.

From results in experiment III, immune duck serum inhibited or neutralized the free leucosis agent as contained in recentrifuged blood plasma of blood from a bird with myeloid leucosis. The uninoculated controls remained free from fowl leucosis during the 10-month period.

This experiment using turkey serum was quite comparable to that in which duck serum was used.

In experiment IV, 0.1 cc. of blood from a bird suffering from myeloid leucosis was drawn directly into a syringe containing 2 cc. of immune turkey serum or physiological saline solution. The solutions were mixed and inoculated into chicks immediately, without allowing incubation of the mixture for time of binding. From results of this experiment, immune turkey serum was able to inhibit or neutralize not only the cell-free agent but the cell-bound agent as well. The use of sodium citrate, heparin or potassium oxalate solutions as anticoagulants, as used in experiment I, has no detrimental effect on affected blood used in experiments.

To determine whether the same inhibitory or neutralizing effect could be achieved for lymphoid forms of the fowl leucosis complex, the following experiments were conducted.

Ducks and turkeys were injected in the same manner and with the same dosage and number of inoculations, using a 1:1,000 saline tissue extract of an ischiatic nerve of a pronounced case of neurolymphomatosis, or neural type of the fowl leucosis complex, and a Berkefeld filtrate of the same tissue. The filtrate was passed through a Berkefeld N filter at a pressure of 381 mm. of mercury.

Nerve tissue was used as there is a quite general agreement as to transmissibility of this form. The ducks and turkeys were bled at the same periods as in the case of myeloid leucosis experiments, and the serum was treated as mentioned in the previous experiments.

The results obtained in experiment V indicate that the causative agent of the neural type of the fowl leucosis complex was neutralized or inhibited by serum from ducks injected with tissue extract from an affected nerve. Similar results were obtained with serum from injected turkeys.

Results of experiment VI indicate that serum from turkeys immunized with the lymphoid type of fowl leucosis was able to inhibit or neutralize the agent of lymphoid leucosis in a cell-free Berkefeld filtrate of the neural type of the fowl leucosis complex. The uninoculated controls of experiments V and VI remained free from infection during the 12-month duration of the experiment.

In order to determine whether cross immunity or inhibitory effect could be achieved between myeloid leucosis and neurolymphomatosis gallinarum, 0.2 cc. of oxalated blood from a chicken suffering with myeloid leucosis was mixed with 4 cc. of serum from ducks that had been given 15 intramuscular injections of 4 cc. each of tissue extract from a nerve of a case of neurolymphomatosis gallinarum. The mixture was placed in the incubator at 37 C. for 2 hours and then inoculated intracardially into 5-week-old chicks.

The results of experiment VII indicate that immune serum from ducks, produced by a series of injections with nerve tissue from neurolymphomatosis gallinarum lesions, was able to inhibit or neutralize the infective agent of the myeloid leucosis type of the fowl leucosis complex, as well as the infective agent of neural types of fowl leucosis. The uninoculated control chicks in this experiment remained free from the disease.

Results of experiment VIII show that the serum of ducks, and the same was true of turkeys, capable of neutralizing the agent of myeloid leucosis was also able to inhibit or neutralize the infective agent of neurolymphomatosis gallinarum contained in tissue extract.

In order to determine whether normal duck or turkey serum might contain an in-

EXPERIMENT IV

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.1 cc. Freshly drawn leucosis chicken blood plus 2 cc. Immune serum (turkey) (Individual sample)	10 6 weeks old	0.2 cc. Intracar- dially	2	10 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.1 cc. Freshly drawn leucosis chicken blood plus 2 cc. Physiological saline solution	10 6 weeks old	0.2 cc. Intracar- dially	9	10 months

EXPERIMENT V

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Nerve tissue (extract) plus 4 cc. Immune serum (duck) (Pooled sample)	20 5 weeks old	0.2 cc. Intracar- dially	3	12 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Nerve tissue (extract) plus 4 cc. Physiological saline solution	20 5 weeks old	0.2 cc. Intracar- dially	13	12 months

EXPERIMENT VI

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Nerve tissue (Berkefeld filtrate) plus 4 cc. Immune serum (turkey) (Pooled sample)	20 8 weeks old	0.2 cc. Intracar- dially	5	12 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Nerve tissue (Berkefeld filtrate) plus 4 cc. Physiological saline solution	20 8 weeks old	0.2 cc. Intracar- dially	12	12 months

EXPERIMENT VII

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Oxalated myeloid leucosis blood plus 4 cc. Immune serum from ducks immunized by lymphoid leucosis (Pooled sample)	20 5 weeks old	0.2 cc. Intracardially	4	12 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Oxalated myeloid leucosis blood plus 4 cc. Physiological saline solution	20 5 weeks old	0.2 cc. Intracardially	16	12 months

EXPERIMENT VIII

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Neural tissue extract plus 4 cc. Immune serum from ducks immunized by myeloid leucosis material (Pooled sample)	20 6 weeks old	0.2 cc. Intraperitoneally	5	12 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.2 cc. Neural tissue extract plus 4 cc. Physiological saline solution	20 6 weeks old	0.2 cc. Intraperitoneally	12	12 months

EXPERIMENT IX

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.1 cc. Myeloid leucosis plasma plus 2 cc. Oxalated blood from hens that survived inoculation (Pooled sample)	10 6 weeks old	0.2 cc. Intracardially	2	12 months

INOCULATED CONTROLS

INOCULUM	NO. OF CHICKS	DOSAGE	NO. OF TAKES	LENGTH OF EXPERIMENT
0.1 cc. Myeloid leucosis plasma plus 2 cc. Physiological saline	10 6 weeks old	0.2 cc. Intracardially	7	12 months

hibitor for the fowl leucosis agent, 1 part of leucosis blood from myeloid leucosis chickens was mixed with 20 parts of normal duck serum and normal turkey serum. The mixture was inoculated intracardially in 0.2 cc. doses into 8-week-old susceptible chicks. The normal, untreated serum did not manifest any neutralizing or inhibiting power, as 70 per cent of the inoculated chicks died or showed blood smears indicative of myeloid leucosis.

Turkeys and ducks were also injected with the same number of injections and in the same way as previously, except that normal hen serum was used. Twenty parts of the serum from the ducks and turkeys were mixed with one part oxalated blood from chickens suffering from myeloid leucosis and inoculated intracardially in 0.2 cc. doses into 6-weeks-old susceptible chicks. The serum from ducks or turkeys treated in this way had no inhibitory power against the fowl leucosis agent.

In nearly all transmission experiments of fowl leucosis, some birds survive the inoculation and may survive several inoculations with leucosis infective material. In order to determine whether survivors of transmission experiments carried an inhibitor to the agent, the blood of six hens from an inoculated group in another experiment were bled. The birds had been inoculated with material of neurolymphomatosis and represented the fifth passage of our strain number 11. The experiment was of 14 months duration when the birds were utilized.

Experiment IX indicates that blood from hens that had survived injections of the agent of fowl leucosis and had resisted repeated injections of the material, inhibited or neutralized the agent of fowl leucosis. This might possibly be true in spontaneously recovered cases, and it is quite possible that some birds become affected and never show definite symptoms.

CONCLUSIONS

1) Immunization of ducks and turkeys by repeated injections of leucosis material produced a serum capable of inhibiting or neutralizing the infective agent or infective power of leucosis cells.

2) The immune serums from ducks and turkeys not only neutralized the cell-free agent but the cell-bound agent as well.

3) Immune serums from ducks injected with myeloid leucosis blood were also able to inhibit or neutralize the agent of the neural type of the fowl leucosis complex.

4) Serums from ducks injected with the neural type of fowl leucosis were able to inhibit the development of the myeloid leucosis type.

5) Neutralization of the agent of one type or form of the disease by duck or turkey serum produced by a series of injections of infectious material from lesions of a different form or type of the complex more closely indicates the possibility of a single etiology.

6) Blood from turkeys or ducks that were not injected with material from cases suffering from the fowl leucosis complex did not contain inhibitors for the agent of fowl leucosis.

7) The blood from recovered birds or from birds that failed to take when inoculated with viable material was able to neutralize the agent of fowl leucosis.

Studies on the Etiology and Pathology of Calf Pneumonia

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MOST OF THE EARLY work on calf pneumonia was associated with studies on calf scours. Hagen[1] in 1917 reported a case of bronchopneumonia associated with scours and joint lesions. Carpenter and Gilman[2] studied the etiology of the disease as a separate entity. Smith[3], and Hallman, Sholl and Delez[4] described lesions of a fetal pneumonia associated with *Brucella abortus* infections. Thorp and Hallman[5] in 1939 reported bacteriological findings similar to those obtained by Carpenter and Gilman. Sanders[6] in 1940, described an enzoötic bronchopneumonia occurring among calves in Florida. He attributed these cases to Pasteurella infection. It is evident from the above studies that there is a lack of agreement regarding the etiologic agent of calf pneumonia. For this reason, as well as the persistent occurrence of this disease in some of the large dairy herds in this section, a further study of calf pneumonia was undertaken.

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[1]Hagan, W. A.: The Etiology and Mode of Infection in White Scours of Calves. Cornell Vet., vii (1917), pp. 263-269.

[2]Carpenter, C. M., and Gilman, H. L.: Studies in Pneumonia of Calves. Cornell Vet., xi (1921), pp. 111-119.

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[4]Hallman, E. T., Sholl, L. B., and Delez, A. L.: Observations on the Pathology of Bacterium Abortus Infections. Mich. Agr. Exp. Sta. Technical Bulletin No. 93, (July 1928).

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MATERIALS AND METHODS

The materials used for this work were obtained from 28 cases of calf pneumonia brought to the animal pathology laboratory for autopsy during the last two years. Eighteen of the cases were killed and examined *post mortem*, immediately, the rest in from one to three hours after death. Portions of tissue were saved from the various lobes, depending upon the extent of pneumonia. All tissues were fixed in Zenker's solution, embedded in paraffin, and sections seven microns thick were stained routinely with hematoxylin and eosin. Heidenhain's modification of Mallory's aniline blue stain was used in several instances as a differential stain. Three series of serial sections were prepared from previously sectioned material.

Bacteriological studies were made on 24 of the cases which came for autopsy. From these cases, 112 primary isolations from the lungs, bronchial lymph nodes, and heart blood were made on blood agar, prepared by adding 10 per cent bovine blood to a veal infusion base prepared in the usual manner. For subcultures, chocolate agar and blood agar supplemented with either vegetable or yeast extract were used. The pH of these mediums was adjusted to 7.4 to 7.6. Sterilization was at 120 C. for 20 minutes. Yeast and vegetable extracts as well as blood were added aseptically after sterilization.

BACTERIOLOGY

The morphology and serology of the cultures isolated in this study indicates that the bacteria found may be divided into two groups as follows: (1) gram-negative nonmotile rods; and (2) pleomorphic gram-variable coccobacilli. The organisms in these two groups occurred singly and in combination with each other.

The first group, comprised of 70 cultures of gram-negative rods, was encountered more frequently than the second, being present in pure culture in five of the calves examined, and associated with other organisms in 14 of the calves. In most instances, the growth from primary isolations after 24 hours incubation on blood agar was scant, the colonies were small and dew-drop in appearance. After 48 hours, the colonies

Fig. 1.—Lungs from a typical case of calf pneumonia of two weeks duration. Note the extent of pneumonia in the diaphragmatic, apical, and cardiac lobes and the enlarged bronchial lymph node. (1/4 actual size).

spread and joined each other and formed a watery colony covering much of the surface of the plate. The consistency of the colonies was quite tenacious, a characteristic which increased with the age of the culture. This group of organisms grew better on chocolate blood agar, forming fine bead-like colonies which later became a grayish-white tenacious mass. It was noted that the longer these cultures were maintained on artificial culture mediums the greater the tendency for such slimy, tenacious colonies to appear earlier during growth. Both yeast and vegetable extract stimulated the growth of most of these cultures. Gram's stain of primary isolations showed gram-negative straight and curved rods with many continuous filaments and short coccoidal forms (fig. 5). These filamentous forms frequently covered the entire diameter of the microscopic field (fig. 6), but in subcultures showing an early development of tenacious growth, these filaments did not appear. On account of the difficulty encountered in culturing these organisms, biochemical characteristics were not readily determined. Two cultures in this group formed some indol, but no reaction was noted in serum-sugar broths or litmus milk to which blood had been added. Two cultures in this group were somewhat pathogenic for guinea pigs, rabbits, and

mice, producing some congestion of the lungs in four days when given intranasally and intratracheally in the rabbit (fig. 6).

The second group of bacteria, which consisted of 26 cultures, comprising the pleomorphic gram-variable coccobacilli were slow-growing organisms (fig. 7).

They produced small, clear, moist colonies which did not become especially tenacious; they did not produce indol and were nonpathogenic for calves, rabbits, guinea pigs and mice when given intranasally and intratracheally in proportionate amounts. The difficulties in determining the biochemical tests in group 1 were encountered again in this group. Sixteen other cultures of organisms were isolated, six of which were *Escherichia communior*. The remainder consisted of seven cultures of staphylococci, two of streptococci, and one of mold. These were considered as contaminants or secondary invaders because of the preponderance of the other types or organisms isolated.

As previously mentioned, due to the inert growth qualities of these organisms, difficulty was encountered in repeated failures to act upon special mediums used for differentiating bacteria. Growth definitely occurred in those mediums to which blood had been added. No action was demonstrated on lactose, maltose, sucrose, mannose, inulin, dextrose, salicin, and arabinose. No growth was obtained in gelatin, plain agar, potato medium, litmus milk, and only in two cultures was there any evidence of indol production. These characteristics were observed only in the case of the first two groups of cultures.

The result of attempts to reproduce the disease by the injection of gram-negative rods isolated from spontaneous cases of calf pneumonia are shown in table 1. Only 6 of the 16 calves showed any evidence of pneumonia or bronchitis, 4 of which were injected with 1 cc. of a suspension of the organisms intratracheally. The other 2 were injected with 2 cc. intranasally, and 1 calf was injected intravenously with 4 cc. of the suspension. All suspensions were adjusted to an approximate nephelometer reading of 2. In the cases in which a recent pneumonia was observed, it should be noted that the gross lesions were not extensive

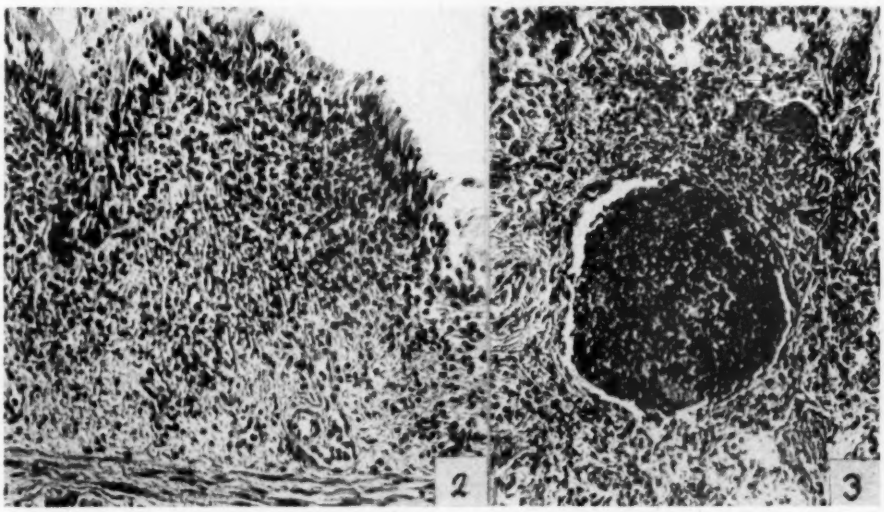


Fig. 2.—Section of the mucosa of one of the small bronchi showing a recent infiltration of polymorphonuclear leucocytes and mononuclear phagocytes into the mucosa. There is some necrosis of the epithelium. (x 150).

Fig. 3.—Section showing a severe bronchiolitis with a necrotic exudate in the lumen, complete desquamation of the epithelium and necrosis of some components of the stroma. (x 150).

and were scattered throughout the apical lobes. The 2 cases showing a bronchitis did not show any lesions other than a marked inflammation of the bronchi. Inconclusive results evidenced in table 1 would suggest that perhaps a virus is associated with this organism in spontaneous cases of calf pneumonia.

TABLE I.—The Results Observed from the Inoculation of Pure Cultures into Calves which Were Slaughtered 10 Days after Inoculation.

CALF NO.	CULTURE	AGE WEEKS	ROUTE OF INOCULATION	AUTOPSY FINDINGS	CULTURES REISOLATED
1	946–1	2	Intravenous	Negative	+
2	946–1	2	Intranasal	Negative	—
3	1026–4	3	Intranasal	Negative	—
4	1026–4	4	Intratracheal	Recent Pneumonia	+
5	T1	2½	Intravenous	Negative	—
6	T1	3	Intranasal	Negative	—
7	1052	1½	Intranasal	Negative	+
8	1052	2	Intranasal	Recent Pneumonia	+
9	T1	3	Intratracheal	Severe Bronchitis	+
10	T1	4	Intratracheal	Recent Pneumonia	+
11	1238	2	Intranasal	Negative	—
12	1238	2	Intratracheal	Recent Pneumonia	+
13	T3	3	Intranasal	Negative	—
14	T3	4	Intranasal	Negative	—
15	T3	4	Intranasal	Negative	—
16	T4	5	Intranasal	Slight Bronchitis	—

The serological reactions of the first two groups of bacteria and known cultures were made by actively immunizing rabbits with 10-12 cc. of killed antigen, followed by 10-12 cc. of living bacteria, to obtain the necessary antiserums. The killed antigen was prepared by washing agar slants with 0.5 per cent tricresolized saline, centrifuging, pouring off the supernatant liquid and resuspending in more tricresolized saline. The suspension was allowed to stand in the refrigerator for 24 to 48 hours and then diluted with normal saline to a McFarland nephelometer standard number 4. The suspension was then tested for sterility. The live antigen was secured by washing a 24-hour growth off a blood-agar slant with normal saline. The injections, with both killed and living antigens, were given daily beginning with 0.25 cc. and increasing the dosage to 2 cc. The agglutinations were determined by placing 0.5 cc. of saline in each tube and adding 0.5 cc. of 1 : 10 dilution of serum to the first tube, mixing and taking out 0.5 cc. and adding to the next tube for the entire series, leaving the last tube for a control. Then 0.5 cc. of antigen which had been prepared by washing off a 24-hour growth from a blood-agar slant was added. These cells were ground in a small mortar with absorbent cotton and diluted to McFarland nephelometer numbers 1 to 2. The tubes were incubated for 6 to 12 hours in the 37 C. incubator and placed in the refrigerator at 6 C. for overnight and then read.

Table II shows the titres obtained when five of these gram-negative rods (referred to by symbol T) were used as antigens and agglutinated with homologous and heterologous serums, as well as with three representative pleomorphic forms of the second group (symbol P) and serums from known cultures of the genus Hemophilus and *Pasteurella avicida*.

Table II shows that a distinct antigenic relationship exists between the five different gram-negative rods (T_1 to T_5), in that significantly high titres were obtained in the cross agglutination reactions except where the homologous titres were not especially high. The titres obtained upon agglutinating the gram-negative rods with sera prepared from pleomorphic forms does

not appear significant. The agglutinating titres ranging from 1 : 320 to 1 : 640, obtained when these gram-negative rods were agglutinated with *Hemophilus suis* serums, indicated a relationship between these forms. No relationship was shown between these gram-negative forms and the culture of *Pasteurella avicida* employed.

ported elsewhere[7]. This consisted briefly of a bilateral pneumonia in all or a major portion of the apical and cardiac lobes of the lungs (fig. 1). With the exception of three very acute cases, the anterior lobes were always very atelectatic and in a stage of gray hepatization. Scattered throughout the anterior lobes, both on the surface and

TABLE II.—Agglutination Reactions

	Pleomorphic Group			Typical		Gm-Neg. Rod Group					
Antigen	P7	P8	P9	T5	T3	T2	T1	T6	H. inf. Am. type Culture	H. inf. suis	H inf. Harvard Strain	P. Avicida
T1	40	20	0	160	2560	2560	1280	2560	0	640	0	80
T2	20	20	0	160	2560	2560	640	1280	20	640	0	40
T3	0	20	0	0	2560	2560	1280	1280	0	320	80	40
T4	0	0	0	80	640	2560	640	640	0	320	0	0
T5	0	0	0	640	640	640	320	1280	0	640	40	0
P. avicida	0	40	0	0	40	30	20	0	0	40	0	2560
H. inf. suis	0	0	0	80	160	320	160	160	320	2560	640	0

CLINICAL AND GROSS PATHOLOGY

The first manifestation of the disease was a dry cough accompanied by considerable lacrimation, soon followed by a mucopurulent nasal discharge. There was an increase in the respiratory rate and breathing was shallow. Soon after the onset of the cough there was a rise in body temperature varying from 103.8 to 105 F. The cough usually became loose on the third or fourth day, at which time the calf usually lost its appetite.

The gross pathology in practically all the cases observed was the same as that re-

upon section, there were a variable number of small abscesses. About 1/3 to 2/3 of the diaphragmatic lobes were usually in a state of red hepatization with the exception of a small area immediately adjacent to the cardiac lobe which usually showed a grayish hepatization. The mediastinal lobe was consolidated in practically all cases, and for the most part its gross pathology resembled the anterior lobes. The cut surfaces of the areas of longer duration were characterized by a number of small abscesses and a mucopurulent material exuding from the small bronchi. The large bronchi were always

CAPTIONS TO ILLUSTRATIONS ON OPPOSITE PAGE

Fig. 4.—Section through an alveolus showing the mononuclear phagocytes of the aveolar exudate and two giant cells. (x 950).

Fig. 5.—Film from 24-hour growth of primary isolation of typical gram-negative rods. Note the long filamentous forms. (x 950).

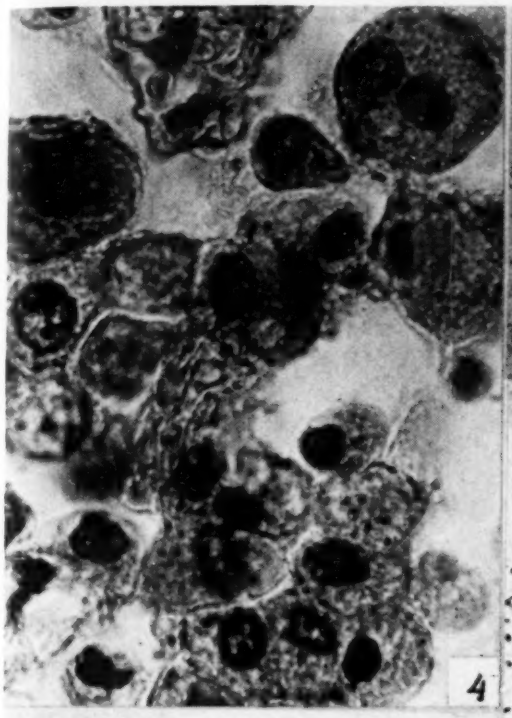
Fig. 6.—Film from 24-hour growth of primary culture reisolated from lung of rabbit injected intratracheally, Gram's stain. (x 950).

Fig. 7.—Film from 24-hour growth of gram-variable bacilli characteristic of pleomorphic group. (x 950).

Fig. 8.—Film from 24-hour chocolate agar culture showing predominantly typical gram-negative rods and some filamentous forms. (x 950).

Fig. 9.—Film from above 24-hour culture showing capsules, Churchman capsule stain. (X 950).

Fig. 10.—Film from 24-hour cholocate agar culture of Hemophilus suis showing more typical forms, alkaline methylene blue. (x 950).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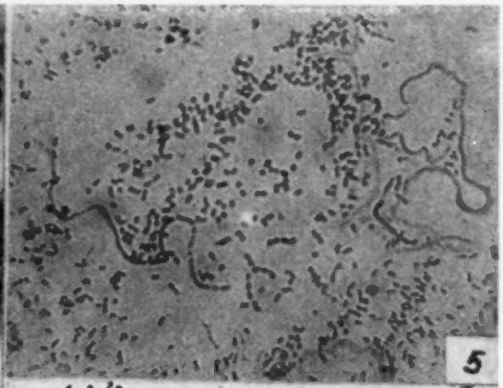
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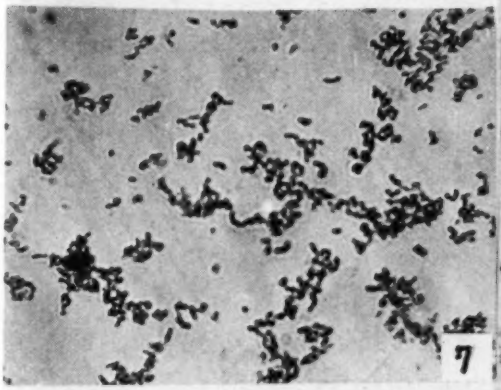
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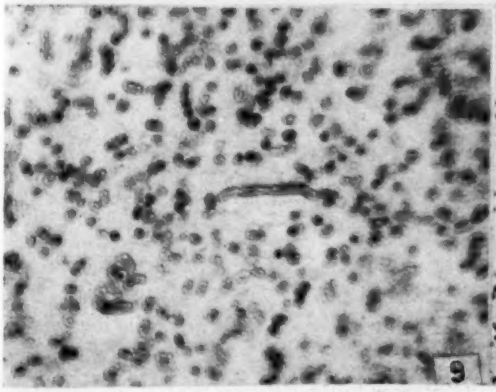
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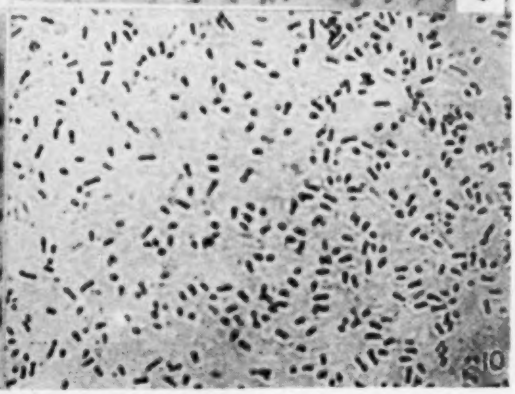
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highly inflamed in that portion near the trachea and contained a variable amount of grayish-white mucopurulent material. Enlarged and edematous mediastinal and bronchial lymph nodes were always observed. A pleurisy was observed in 18 of the 28 cases.

HISTOPATHOLOGY

The microscopic examination of sections of the pneumonic lungs of calves revealed lesions of a typical interstitial bronchopneumonia. The mucosa of the larger bronchi was highly congested and infiltrated with polymorphonuclear leucocytes and mononuclear phagocytes accompanied by varying degrees of desquamation of the bronchial ephithelium (fig. 2). The small bronchi and bronchioles were filled with a cellular exudate consisting of polymorphonuclear leucocytes and some few mononuclear phagocytes; in addition the lumen of the bronchi and bronchioles contained many desquamated epithelial cells. The most severe lesions of the bronchial tree were in the terminal and respiratory bronchioles. The extent of desquamation of the epithelium of the bronchioles varied in different cases. The stroma was usually infiltrated with mononuclear phagocytes and some polymorphonuclear leucocytes. In some areas of longer duration, the epithelium of the respiratory bronchioles was entirely destroyed and the lumen was filled with a mass of necrotic material (fig. 3). The stroma of these bronchioles contained many mononuclear phagocytes with pyknotic nuclei and a small number of proliferating fibroblasts. The surrounding peribronchial tissues contained many mononuclear phagocytes and dead and disintegrating polymorphonuclear leucocytes. Upon cross section, many of these bronchioles gave the appearance of early abscesses.

The alveoli immediately adjacent to the bronchioles were filled with a cellular exudate consisting mostly of mononuclear phagocytes and some polymorphonuclear leucocytes. The alveoli more distant from the bronchioles usually contained a fibrinous or a serous exudate in which there were small numbers of mononuclear phagocytes and polymorphonuclear leucocytes. Capillaries of the aveolar walls were highly congested and the wall appeared thickened by an increase of mononuclear phagocytes in that area.

In cases of calf pneumonia, diagnosed clinically and grossly as acute, large numbers of alveoli were found filled with a cellular exudate which consisted almost entirely of mononuclear phagocytes and giant cells (fig. 4). It was observed upon the study of serial sections that the terminal and respiratory bronchioles bordered on abscess formation as a result of the severe bronchiolitis. In areas where the bronchiolitis was of a more acute nature and not as extensive, but where a necrosis and some bronchiole injury had taken place, the surrounding alveoli did not contain the large number of mononuclear phagocytes and giant cells mentioned previously.

A thickening of the pleura was always observed in the more chronic cases.

DISCUSSION

The results of this study suggest that the gram-negative, rod-shaped bacterium isolated in 19 of the 24 cases of calf pneumonia, examined bacteriologically, are closely related to members of the genus Hemophilus.

These gram-negative rods, which require the addition of blood to the medium for growth and the growth of which is enenhanced by vegetable or yeast extracts, show nutritional requirements similar to the hemophilic organisms. Hemophilus members were early classified on their need for the so-called X and V growth factors, either alone or in combination, supplied from blood, yeast, and vegetable extracts.[7] Morphologically, the cells of these gram-negative rods (fig. 8) resemble the members of the genus Hemophilus; in each are observed curved rods, filaments and coccoid forms. In some of the typical gram-negative rods, capsules were demonstrated (fig. 9).

Serologically, a definite relationship was

[7]Thjotta, T., and Avery, O. T.: Growth Accessory Substances in the Cultivation of Hemophilic Bacilli. J. Exp. Med., xxxiv, (1921), pp. 97-114.

shown to exist between these gram-negative rods and *Hemophilus suis* (fig. 10). The failure of certain of the gram-negative forms to reach a very high agglutination titre with *Hemophilus suis* serums is similar to the findings of Lewis and Shope[8] in their studies of swine influenza.

The fact that the efforts to reproduce the disease in calves injected with the gram-negative rods isolated from spontaneous cases of calf pneumonia failed in most instances suggests the possibility that a virus may be involved. It is an established fact in human and swine influenza that the virus and the organisms are associated with each other in producing a pneumonia. The virus alone produces only a mild inflammatory reaction but secondary organisms associated with it may then produce a pneumonia. Further investigations are in progress to determine the presence of a virus in calf pneumonia, the rôle of the pleomorphic forms isolated, and agglutination absorption studies.

The histopathology of these cases was very similar to that previously reported by workers in other parts of the country. McCallum[9], in his work on influenzal pneumonia, states that both the hemolytic streptococci and the influenza bacilli of Pfeiffer will cause an interstitial bronchopneumonia in which there is a difference in the microscopic picture of the two types. On this basis he makes the statement that the anatomical picture of pneumonia is so characteristic that it is a good indication of the bacteria concerned. In this type of pneumonia there is a marked hyperplasia of the so-called epithelial cells of the alveoli which produce masses that appear as giant cells. Lewis[10] by the use of cultures of cells, showed that the size of the mononuclear phagocyte depends upon its opportunity to stuff itself with foreign material and increase in size.

In the bronchopneumonia observed in the calf, there was considerable foreign material in the alveoli, such as fibrin, dead polymorphonuclear leucocytes, and lymphocytes, which would probably account for the large number of mononuclear phagocytes around the periphery of the lesion produced by the severe injury to the terminal and especially the respiratory bronchioles. It was observed by the study of serial sections of practically entire lobules and their affected bronchioles that the number of mononuclear phagocytes and giant cells observed at the periphery of those areas surrounding the necrotic and severely injured bronchioles was in proportion to the degree of injury. These lesions were observed in those cases which would be considered as acute cases of pneumonia clinically.

[8]Lewis, P. A., and Shope, R. E.: A Hemophilic Bacillus from the Respiratory Tract of Infected Swine. J. Exp. Med., liv (1931), pp. 361-371.
[9]MaCallum: Textbook of Pathology, 7th ed., W. B. Saunders Co., Philadelphia, (1940), p. 779.
[10]Lewis, W. H., and Lewis, M. R.: As quoted by MacCallum: Textbook of Pathology, 7th ed., W. B. Saunders Co., Philadelphia, (1940), p. 161.

Summary

1) Histopathological studies were made on 28 cases of calf pneumonia. Bacteriological studies were made upon 24 of the above cases.

2) Hemophilic-like bacteria were consistently isolated from the cases examined. Attention has been called to the marked similarity of the gram-negative organism isolated to *Hemophilus suis*.

3) Attempts to introduce pneumonia in calves by inoculations of the organisms did not meet with consistent or conclusive results.

4) Histopathological studies reveal a characteristic bronchopneumonia similar to that reported elsewhere[5]. However, the degree of injury to the bronchioles appeared more severe in the cases that were observed in this study.

5) The numerous giant cells and large mononuclear phagocytes observed in cases of acute pneumonia appear to be in proportion to the extent of injury and necrosis in the terminal and respiratory bronchioles.

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Distemper Inclusion Bodies

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IN SOME of the virus diseases, intracellular bodies are of more or less constant occurrence in certain tissues. Among the better known are the Negri bodies in the brain cells in rabies, the Guarnieri bodies in epidermal cells in variola and vaccinia, and the Bollinger body in fowlpox. These bodies are generally characterized and differentiated by their acidophilic or eosinophilic affinities when specimens are treated with suitable, combined stains.

In certain other virus diseases, cell inclusions, cytoplasmic and intranuclear, may also be found, differing somewhat from the types mentioned but with similar staining reactions. Their occurrence has been observed and reported, by not a few investigators, in the tissues of animals infected with the viruses of encephalitis, choriomeningitis, and other neurotropic virus diseases, and in both the viscerotropic and neurotropic forms of distemper. Their relationship to the viruses with which they appear to be associated, and their significance as an indicator of the amount, concentration and distribution of the virus present in the tissues, and as a means of microscopical diagnosis, are frequently mentioned and sometimes asserted in the literature but, nevertheless, are regarded by many as problematical. Certainly, the bodies seen in some virus diseases are indistinguishable from those seen in others; and in some cases of the same disease the bodies may be found without great difficulty, while in others a prolonged search does not reveal them. In the course of distemper studies extending over a period of years, we have searched at different times for inclusion bodies in the tissues of animals experimentally infected with distemper virus, as well as in animals dead or dying from distemper in natural outbreaks of the disease on fur farms.

Procedure and Staining Methods.—The tissues examined include brain, kidney, urinary bladder, lung, trachea, liver, spleen, stomach and intestine. Smear and impression preparations fixed in absolute alcohol were made of certain tissues, such as brain and the lining membranes of the bladder and trachea, in addition to sections fixed in saline formol solution and embedded in paraffin. The routine stains were Williams' modification of Van Gieson's method for smears, and Harris' hematoxylin and Le Mesurier's triple eosin mixture for sections.

Description of Distemper Inclusion Bodies.—The bodies are readily recognized in preparations stained with Le Mesurier's triple eosin. In size and shape they vary from small granules to round or oval bodies several times larger than a red blood corpuscle. With proper differential staining technique, the red blood cells are colored a pale red or pink with a yellowish or orange tinge, the inclusion bodies a deeper and purple or bluish red comparable to the staining of Negri bodies. The inclusions are intracellular and nearly always cytoplasmic. In some cells, as many as seven or eight small eosinophilic inclusions may be present, in others, only one; or, a single large body may fill the greater part of the cell cytoplasm so that the entire cell is seemingly composed of its nucleus and the inclusion body. A minute, bright, uncolored point in the center of the body, a vacuole, or a faint halo surrounding the body is sometimes apparent and suggestive of refractile properties.

INCLUSION BODIES ASSOCIATED WITH DISTEMPER VIRUSES IN THE TISSUES OF INFECTED FERRETS

a) *Examination of Brain Tissues.*—In the course of propagating two strains of distemper virus by animal passages (July, 1933 to June, 1934), 42 ferrets inoculated with virus of canine origin and 33 with fox

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virus, together with 8 ferrets in cohabitation with the infected animals, developed typical distemper and died of the disease or were killed *in extremis*. The brain only of each of these 83 ferrets, in sections and smears, was examined for cell inclusions. Eosinophilic granules were noted in smear preparations, but not in the sections, of the brain cells of 5 ferrets. The granules occurred in only a few cells in one or two of the dozen smears made from each brain and probably bear no significance in respect to distemper inclusion bodies (table I).

TABLE I.—Inclusion Bodies in Brain Tissues

		Ferrets dead or dying from distemper					
VIRUS ORIGIN	INFECTED BY	TOTAL NUMBER	DIED (D) OR KILLED (K)		BRAIN TISSUES EXAMINED FOR CELL INCLUSIONS		
					+	—	?
Dog	Inoculation	42	d.	10		10	..
			k.	32		31	1
Fox	Inoculation	33	d.	8		7	1
			k.	25		23	2
Dog and Fox	Cohabitation	8	d.	3		2	1
			k.	5		5	..
		83		83		78	5*

*Granules only. Small, refractile, eosinophilic, irregular contour, in Purkinje type cells, and of very doubtful significance.

b) *The Kidney and Urinary Bladder.*—Specimens of kidneys and urinary bladders were collected from 17 ferrets inoculated with distemper virus "P" (dog, fox and mink strains pooled). Six of the ferrets were inoculated with the virus after passage in serial order through 86 ferrets, 7 ferrets were inoculated with the eighty-seventh passage, and 4 with the eighty-eighth passage virus.

Definite eosinophilic bodies were found in 11 of the 17 ferrets (64.7%). In 8 instances the bodies were present in both kidney and bladder tissue, in 2 instances in the bladder alone, and in 1 animal they were found only in the kidneys. Questionable bodies were present in 2 ferrets. These were found in both the bladder and kidney tissues in 1 animal, but only in the kidney in the other. In the tabulated summary, table II, it will be noticed that 8 of the ferrets died of the disease and that definite inclusion bodies were found in 6 (75%), and indefinite eosinophilic granules in the other 2. The other 9 animals were killed *in extremis*, and inclusion bodies were found in 5 (55.5%).

c) *The Tissues in Which Inclusion Bodies May Be Found and the Day of Their First Appearance After Virus Inoculation.*—Eight normal ferrets, removed from a healthy colony and never exposed to infection, were inoculated with ferret spleen-tissue distemper virus "P". At 2-day intervals, from the second to the fourteenth day following infection, one of the ferrets was killed; the last of the series died from distemper on the fifteenth day.

The incubation and symptomatic periods, approximately 10 and 5 days respectively, were typical of ferret distemper. The first appearance of inclusion bodies was on the tenth day in ferret 5, just when clinical symptoms were about to appear. They were found in each of the 3 remaining ferrets, on the twelfth, fourteenth and fifteenth day, respectively, and only in the tissues of the liver, gall bladder, kidneys and urinary bladder (table III).

d) *Absence of Inclusion Bodies in the Tissues of Ferrets Immune to Distemper.*—Eight immunized ferrets were inoculated at the same time and in the same manner

TABLE II.—Inclusion Bodies in Kidney and Bladder Tissues

			Ferrets dead or dying from distemper					
VIRUS ORIGIN	SERIAL PASSAGE	TOTAL NUMBER	DIED OR KILLED	INCLUSION BODIES KIDNEY	INCLUSION BODIES BLADDER	NUMBER OF FERRETS +	NUMBER OF FERRETS —	NUMBER OF FERRETS ?
Dog, fox and mink virus strains pooled into one composite strain "P"	86th	6	Killed	+	+			
			Killed	+	+			
			Killed	—	+			
			Killed	—	—			
			Killed	—	—			
			Killed	—	—	3	3	
	87th	7	Killed	+	+			
			Killed	+	—			
			Killed	—	—	2	1	
			Died	+	+			
			Died	+	+			
			Died	+	+			
			Died	?	?	3		1
	88th	4	Died	+	+			
			Died	+	+			
			Died	—	+			
			Died	?	—	3		1
Total Number		17		9(+)	10(+)	11	4	2

Per cent, 64.7 Positive; 23.5 Negative; 11.7 Questionable.

TABLE III.—Time Period and Tissues in Which Inclusion Bodies Make Their Appearance in Experimental Ferret Distemper

PERIOD	INCUBATION					SYMPTOMS		
Ferret No.	1	2	3	4	5	6	7	8
Day killed after inoculation	2nd	4th	6th	8th	10th	12th	14th	15th*
Tissues examined for inclusion bodies								
Brain	—	—	—	—	—	—	—	—
Trachea	—	—	—	—	—	—	—	—
Lungs	—	—	—	—	—	—	—	—
Stomach		—	—	—	—	—	—	—
Spleen	—	—	—	—	—	—	—	—
Liver	—	—	—	—	+	+	+	+
Gall bladder	—	—	—	—	—	—	+	+
Kidneys	—	—	—	—	+	+	+	+
Urinary bladder	—	—	—	—	+	+	+	+
Adrenals		—	—	—		—	—	—

*Died of distemper.

Virus origin: Dog, fox and mink strains, pooled as composite strain "P."

Serial ferret passage: 106th.

Number of ferrets inoculated: 8 (1 to 8).

Inoculum: 1.0 cc. of 10 per cent ferret spleen suspension.

and dosage as the 8 normal ferrets in the preceding experiment and were killed at the same intervals of 2 days (8th-16th day). No visible clinical reactions in the immune ferrets could be observed and no inclusion bodies could be found in any of the tissues examined.

e) *Search for Inclusion Bodies in the Tissues of Normal Ferrets.*—Eleven ferrets were taken from the healthy ferret colony and killed with chloroform. Inclusion bodies were found in only 1 of the 11 ferrets, and only in the kidney, in small numbers but similar to and indistinguishable from those found in ferrets infected with distemper virus.

The possibility of the inclusion bodies found in this single healthy ferret being associated with distemper can be ruled out since there was not the slightest evidence or suspicion of the disease in the ferret colony from which it was taken, before or after.

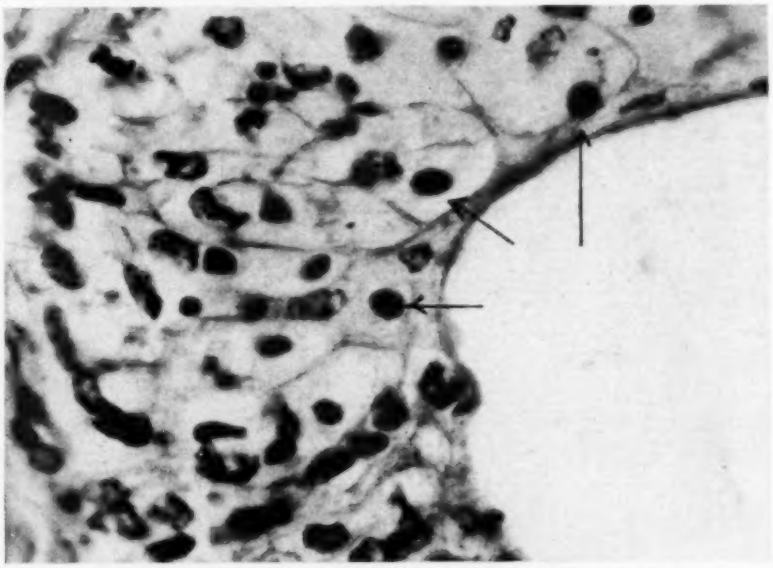
f) *Ferrets Infected with Distemper Virus from Mink.*—This virus, identified as strain "C", was originally obtained from a natural outbreak of distemper on a mink farm and has been separated in the laboratory, by Heath (1940) into two substrains (a) neurotropic, derived from adult mink and causing nervous symptoms only in ferrets, and (b) viscerotropic, from mink kits, causing the usual characteristic catarrhal exudates from eyes and nose hyperemic conditions, lip vesicles, etc.

With the neurotropic strain, definite inclusion bodies were found in each of the 2 ferrets of the first passage and in 1 of the 3 ferrets of the fifth passage; no inclusion bodies were found in any of the 12 ferrets of the sixth passage.

With the viscerotropic strain, 3 of the 6 ferrets of the seventh passage showed inclusion bodies.

Thus, cell inclusions were present in certain tissues of 6 of the 23 ferrets in this series, dead or dying from distemper, in kidney and urinary bladder of 3, kidney and liver of 1, bladder and liver of 1, and bladder only of 1 (table IV).

g) *Foxes Immune to Distemper Virus.*—Thirty foxes of proven immunity to distemper virus received a further test inoculation of virus. Eight additional im-



—After Watson and Plummer, 1942

Distemper Cell-Inclusion Bodies

TABLE IV.—Inclusion Bodies in Ferrets Dead or Dying from Distemper Following Inoculation of Mink Strain of Virus

Ferrets Serial Passage	Serial Number	Brains	Trachea	Lungs	Spleen	Liver	Gall bladder	Kidney	Urinary bladder	Adrenals	Day after inoculation Killed *in extremis* or Died from distemper
'a'	1							+	+		k.
1st	2							+	+		k.
	3					—		+	+		k.
5th	4					—		?	—		k.
	5					—		?	?		k.
	6	—	—	—	—	—	—	—	—	—	d.
	7	—	—	—	—	—	—	—	—	—	k.
	8	—	—	—	—	—	—	?	—	—	k.
	9	—	—	—	—	—	—	—	—	—	k.
6th	10	—	—	—	—	—	—	—	—	—	k.
	11	—	—	—	—	—	—	—	—	—	k.
	12	—	—	—	—	—	—	—	—	—	k.
	13	—	—	—	—	—	—	—	—	—	d.
	14	—	—	—	—	—	—	—	—	—	d.
	15	—	—	—	—	—	—	—	—	—	k.
	16	—	—	—	—	—	—	—	—	—	k.
	17	—	—	—	—	—	—	—	—	—	k.
	18		—	—	—	—		—	+		
'b'	19		—	—	—	—		?	—		
	20		—	—	—	—		—	—		
7th	21		—	—	—	+		—	+		
	22		—	—	—	—		—	—		
	23		—	—	—	+		+	—		
Inclusion bodies+						2		4	5		in 6 ferrets
Inclusion bodies?								4	1		in 4 ferrets
Inclusion bodies—		12	18	18	18	19	12	15	17	12	in 13 ferrets

Virus "C": *'a'*, Neurotropic substrain from *adult* mink. *'b'*, Viscerotropic substrain from mink *kits*.

mune foxes were further exposed to infection by cohabitation with infected animals. Each of these 38 immune foxes was killed, after a varying time interval following inoculation and exposure, as shown in table V.

The viruses given to test the immunity, and with the object of possibly causing the appearance of cell-inclusion bodies, were lethal for ferrets, which succumbed to typical distemper following the usual incubation and symptomatic periods. However, none of the foxes exhibited any clinical manifestations of distemper but carried their immunity throughout.

Specimen material of the brain, trachea, lungs, stomach, spleen, kidneys, adrenals, liver, and the gall and urinary bladders were collected from each animal immediately after it was killed. All proved negative on close and long search for inclusion bodies.

h) *Enzoötic Diseases on Fur Farms.*—Heavy losses occurring in the course of spreading outbreaks of disease on isolated fox and mink farms are not infrequently reported. For the most part, these are attributed by the owners or managers, as well as by attending veterinarians, to distemper when no other cause or likely factor is readily discernible, even though the clinical evidence to support a diagnosis of distemper may be very scanty. In a number of these, the tissues of one or more affected animals in each outbreak were searched for cell-inclusion bodies, and ferrets were in-

oculated with the same specimen material to determine the presence or absence of distemper virus.

The laboratory findings made in respect to 6 natural enzoötics, 3 occurring on fox farms and 3 on mink farms, are shown in table VI.

TABLE V.

IMMUNE FOXES	TEST INOCULATION AND EXPOSURE	SUBSEQUENT INTERVAL (KILLED)
9	Virus "C" Subcutaneous	52-54 days
3	Virus "C" Subcutaneous	6 months
5	Virus "C" Intracerebral	52-54 days
4	Virus "C" Intracerebral	6 months
5	Virus "P" Subcutaneous	54-56 days
4	Virus "P" Intracerebral	54-56 days
5	Exposed by cohabitation with infected foxes	52-56 days
3	Exposed by cohabitation with infected foxes	4 months
38		

The history and reported symptoms in enzoötics 1, 4 and 5 were more or less indicative of distemper, ferret inoculations were positive, and cell inclusions were found in the kidneys or bladder of 6 of the 13 mink and foxes examined. Enzoötics 2 and 3 were not suggestive of distemper, the former being characterized by gastroenteritis, fatty degeneration of the liver and, in some cases, pneumonia, and the latter by skin hypersensitivity, paralysis, and sudden death. Inoculation tests of ferrets proved negative for distemper. Nevertheless, cell-inclusion bodies were found in kidney or bladder tissues of 2 of the 7 mink carcasses examined.

Only 1 fox, representing enzoötic 6, was examined. No inclusion bodies could be found in the tissues, but ferret inoculation was positive for distemper.

i) *Spontaneous Outbreaks of Distemper in Ferret Colonies.*—In the course of raising large colonies of ferrets for experimental purposes, over a period of more than 10 years, two spontaneous outbreaks of distemper occurred, one in the year 1938, the other in 1941. The entire colonies were nearly eradicated by the disease. In the first outbreak, 27 ferrets dead or dying from distemper were examined and inclusions were found in 21 (77.7%), and in the second outbreak 15 ferrets were examined and inclusion bodies found in 4 (26.6%), in kidney and/or bladder tissues.

SUMMARY

Eosinophilic cell-inclusion bodies, as described, were frequently present in certain tissues of ferrets, foxes and mink when these animals were in the end stages of, or dead from, experimental distemper, or in natural enzoötics of the disease.

The inclusions were most frequently seen

TABLE VI.

NATURAL ENZOÖTIC (FUR FARMS) No. ANIMALS EXAM'D	BRAIN	LUNGS	SPLEEN	LIVER	GALL BLADDER	KIDNEY	URINARY BLADDER	STOMACH	INTESTINE	FERRET TEST INOCULATIONS (FOR DISTEMPER VIRUS)
1. Mink 2						—	1			Positive
2. Mink 4			—	—	—	1	—	—	—	Negative
3. Mink 3	—			—	—	—	1		—	Negative
4. Foxes 7				—	—	3	—			Positive
5. Foxes 4		—	—	—	—	1	2		—	Positive
6. Foxes 1		—	—	—	—	—	—			Positive

in the epithelium of the urinary tract (kidney, pelvis and bladder), occasionally in the epithelium of the bile ducts of the liver and of the gall bladder, but never in the tissues of the brain, trachea, lungs, spleen, stomach and intestines.

Cell inclusions were detected in from 25 to 65 per cent of infected ferrets, the percentage varying in groups infected with different virus strains, and in approximately 33 per cent of mink and foxes.

Positive findings could not be made in ferrets killed during the incubative stage of infection, but were obtained in increasing number and percentage as the disease advanced to the final stage and were highest in animals which actually succumbed to it.

The inclusions were almost invariably intracellular and cytoplasmic. Very rarely an inclusion was seen which might have been intranuclear, or only apparently so by its location on or under the cell nucleus.

Only negative findings were obtained in ferrets artificially immunized with distemper viruses and killed in from 2 to 16 days after a later inoculation of virus, and in immunized foxes, similarly treated and killed after time intervals of 50 to 180 days.

Cell inclusions, similar in all respects to those found in 1 of a group of 11 normal healthy ferrets were taken from a colony rigidly isolated from infection, and in 2 mink which died of a disease not caused by distemper.

REVIEW AND DISCUSSION

The presence of cell-inclusion bodies in the pathological picture of distemper has caught the attention of a number of writers. Lentz (1908) described such bodies in the central nervous system of dogs. Sinigaglia (1913) regarded them as diagnostic of dog distemper and comparable to the Negri bodies found in dogs affected with rabies. Dunkin and Laidlaw (1926) reported that eosinophile inclusions may be found in a few of the epithelial cells of the skin and cornea and occasionally in the large vacuolated cells of lung lesions in ferrets infected with dog distemper, but could not be found in the brain tissues of either ferrets or dogs. Slanetz and Smetana (1937) studied an epizoötic virus disease of ferrets, of a nature apparently identical to that of the epizoötic recorded in this paper, and state that the most characteristic and constant histologic feature was the presence of inclusion bodies in the epithelial cells of many organs. These were most frequently and constantly seen in the respiratory tract and in the lining cells of the mucosa of the nose, sinuses, trachea and bronchi, and most of them were cytoplasmic but intranuclear ones also were present. They go on to say that inclusion bodies in the lining cells of the kidney, pelvis and of the urinary bladder were as constantly present as in the respiratory tract, but were never found in gall bladder epithelium, nor in the cells of the fallopian tubes, uterus, ovaries, epididymis, testes, brain, spinal cord or meningi.

These authors, from a comparative study on ferrets of the effects of canine distemper virus and the virus of the ferret disease, conclude that there seems to be no immunologic relationship between the virus of the one and that of the other.

Pinkerton (1940), studying epizoötic distemper on mink farms, has made similar observations in respect to the presence and distribution of inclusion bodies, intranuclear and cytoplasmic, in mink dying from distemper or killed during the course of the disease. He stresses the importance of such findings as a rapid and accurate method for diagnosing distemper, and he also states that the standardization of tissue vaccines by observing the number of inclusion bodies microscopically is much simpler than by estimating virus content by animal inoculations.

Green and his coworkers (1939) describe inclusion bodies in fox encephalitis as always intranuclear and found principally in cells of the vascular endothelium, but never in bile ducts nor in other surface epithelial cells where the inclusions of distemper occur. According to Green (1941) "the distemper virus grows typically in the epithelium making up the mucous membranes and is found in the surface cells of the respiratory tract, in the gastrointestinal

tract, biliary system and the urogenital system."

Wisnicky and Wipf (1941), reporting on 646 fox and mink specimens examined for distemper inclusions, mention the finding of cytoplasmic inclusions, similar in shape and in staining properties to the distemper inclusions, in sectioned material from foxes on ranches with no clinical evidence of distemper, and in bladder and tracheal smears from foxes and mink when distemper in clinical form did not occur. They conclude that distemper inclusion bodies are a valuable aid in the diagnosis of the disease, but that it is necessary to consider fully the history, clinical symptoms, autopsy picture, and inclusion body findings to establish a correct diagnosis.

We can say from our own experiences and findings that intracellular, cytoplasmic inclusion bodies are frequently present in animals naturally and experimentally infected with distemper virus (and, in comparatively rare instances, in animals which have had no contact with distemper and have never been exposed to it) and that the animal tissues containing such bodies are more or less limited to the transitional epithelium of the urinary tract and of the gall bladder and bile ducts.

We can say, further, that the inclusions are more likely to be present and located in animals which are dying or have died from the disease than in animals killed before reaching a moribund state. Even so, in ferrets dead from distemper virus infection, inclusion bodies cannot be demonstrated in 25 per cent or more of the animals; and in foxes and mink the number of negative findings rises sharply and may reach up to 65 per cent. Nevertheless, and this is a fact to be stressed, inoculation of tissues of organs, such as the spleen and lungs, in which no inclusion bodies could be detected, has never proved to be less infective or virulent than kidney and other tissues rich in inclusion bodies. That is also true with Berkefeld filtrates of tissue suspensions, regardless of the presence or absence of cell inclusions in the tissues concerned.

We cannot agree that a tissue vaccine can be standardized nor its potency indicated by the presence and richness of distemper inclusion bodies. We have separately prepared vaccines from the different organs of virus infected animals, and from animals in which inclusion bodies were present and others in which they were absent, and titrated them without detectable differences in immunizing value and efficiency against the homologous viruses, or any evidence of a correlation between the presence, number and abundance of inclusion bodies in any given tissue or organ and the concentration of virus in that tissue.

Distemper viruses as viscerotropic and neurotropic types, with changing characters and pathogenic properties by passage in different species of animal hosts and immunologic differentiations, are now recognized. Accordingly, the origin, strains and types of the viruses productive of distemper-like disease may be important factors in determining the type of disease set up, the tissue reactions, and the occurrence of cell inclusions.

We are inclined to view the presence of these inclusions in animals succumbing to distemper as of some significance as an aid in microscopic diagnosis, but not as absolutely specific and pathognomonic of that disease nor for the identification of the virus. We cannot dismiss the thought that the cell inclusions may be not more than an end product of disease processes, not in themselves constituting virus bodies, elementary or otherwise, but perhaps in the nature of a colloidal or lipoidal product, arising from an interaction between substances in the excretions and exudates and the protoplasm of the epithelial cells lining the passages.

Influence of Diluters, Rate of Cooling, and Storage Temperatures on Survival of Bull Sperm

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IN THE PRACTICE of artificial insemination where maximum use of a bull is desired, the problems of a suitable diluent and proper storage methods become important. Many diluters have been recommended which may be suitable (as a diluent) when the semen is used immediately, but very few have consistently given satisfactory results during storage.

The glucose-phosfate diluters recommended by Milovanov and Selivanova[11] and the sulfate and tartrate diluters developed by Milovanov[10] have been used widely. Although satisfactory results have been obtained with these diluters, when used with fresh semen for immediate insemination or for insemination after a short storage time, they have failed to increase the survival time of bull spermatozoa (Hatziolos[6]). In 1940, Phillips and Lardy[14] developed an egg-yolk, phosfate diluter which has generally given satisfactory results in the storage of bull semen and Salisbury, Fuller and Willett[16] have reported comparable results with a yolk-citrate diluter.

It is generally agreed that semen should be stored at a temperature below that of the body, but recommendations vary greatly. It is possible that the varied recommendations could have resulted from the failure to consider either of two factors:

(1) temperature shock from rapid cooling, Birillo and Puhaljskii[1]; Gladcinova[4]; Chang and Walton[3]; Lasley, Easley and McKenzie[9]; Lasley, Easley and Bogart[8]; and Salisbury[15];

(2) storage temperature, Weber[20]; Komarov and Gladcinova[7]; Camici and Polestra[2]; Hatziolos[6]; and Salisbury.[15] For example, semen cooled gradually, Birillo and Puhaljskii,[1] can be successfully stored at temperatures of 0 to 5 C., Chang and Walton.[3]

The study of sperm survival was based upon motility estimations until Walton and Edwards[19] substituted a measurement of sperm respiratory activity and Lasley, *et al.*,[9] described a staining method for differentiating live and dead sperm as more quantitative methods of evaluation.

The studies reported in this paper are concerned with the influence of diluters, storage temperature, and rate of cooling on the survival time of sperm. In this study of semen diluters, two factors were considered: (1) the relation of the physical characteristics of the diluters to those of the semen under investigation, and (2) an accurate criterion of sperm survival.

MATERIALS AND METHODS

Semen studies were made during the summer of 1941 on Hereford bulls at the San Carlos and Fort Apache Indian Reservations in Arizona, and during the winter of 1941-42 on Shorthorn bulls at the University of Missouri. All of the seven bulls used in this work were mature, appeared normal, and produced semen of good motility. They were fed a good ration in a dry lot. The semen was collected with the artificial vagina, and was held at approximately 25 C. from the time of collection until the experimental procedure began. This interval never exceeded one hour. The staining technic of Lasley, *et al.*[9], (1942) was used for determining the percentage of live sperm in the semen sample because the method has been proved to be both sensitive and accurate. The

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staining mixture was composed of 50 cc. of isomotic phosfate buffer, pH 7.2, 0.4 Gm. water soluble eosin B and 14 cc. opal blue stain (Breslau formula). It was heated to boiling, filtered through a qualitative filter paper and stored in a refrigerator (8-10 C.) to prevent mold growth, but was always warmed to about 25 C. prior to use. The semen smears made in Arizona were dried at room temperature, 25-30 C., but the higher humidity in Missouri made it necessary to dry the smears made there over a hot plate in front of an electric fan. For the determination of the percentage of live sperm in a sample, two smears were made and 500 sperm counted on each.

Special consideration has been given to three physical characteristics of semen and diluters, *i. e.*, pH, buffering capacity and osmotic pressure.

The pH determinations were made with the Beckman glass electrode pH meter.

The degree of H ion change induced by definite quantities of acid or base is a measure of buffering capacity. In determining the buffering capacity, 0.5 cc. volumes of standardized N/100 NaOH or HCl were introduced quantitatively into 1 cc. samples of the semen or diluter under investigation and the resulting change in pH determined with the glass electrode pH meter.

The osmotic pressure measurements were made with a Beckmann molecular weight apparatus and expressed as the degrees centigrade representing the relation of the freezing point of the solution to that of pure water.

RESULTS

Diluters.—The only diluters capable of prolonging sperm survival beyond that in undiluted semen were the phosfate buffer and the yolk-phosfate. The yolk-phosfate diluter was superior to all the others, particularly for the longer storage times. The superiority of this diluter was shown in other studies (data not published) in which a large number of samples were used in artificial insemination.

All the GPC* diluters were markedly harmful to sperm during storage (table 1) as shown by the presence of fewer live sperm immediately after diluting and after storage of 24 and 72 hours. The GPC diluters also differed in physical characteristics from undiluted semen, the phosfate buffer, and the yolk-phosfate diluter (table I and fig. 1).

Although the sulfate and tartrate diluters showed no immediate harmful influence upon the sperm, and their physical characteristics corresponded more closely to those of semen than those of the GPC diluters, semen diluted with them did not store as well as undiluted semen. The relation of these physical characteristics to sperm survival will be considered in the discussion.

The data in table I are averages of four trials. In each of these trials, nine 0.5 cc. samples were taken from a single semen collection. One sample acted as a control while each of the other eight were diluted with one of the various diluents (table I). The semen and the diluter, at a temperature of 25 C., were mixed in the proportion of 1.5 cc. diluter to 0.5 cc. of semen. The small vials of diluted semen were placed in a beaker containing 300 cc. of water at 20 C. and the beaker stored in a refrigerator at 8-10 C. This procedure insured a slow controlled cooling rate. Smears were made immediately after dilution and after storage periods of 24 and 72 hours.

Influence of Rate of Cooling on Undiluted Semen.—Cooling at a rapid rate resulted in a low percentage of live sperm even when the semen was only stored for nine hours. The most rapid cooling rate for undiluted semen that could be considered at all satisfactory was that of 5 C. steps at one hour intervals.

In the studies on rate of cooling, a standard 0.5 cc. volume of undiluted semen and semen diluted 1 : 3 with yolk-phosfate buffer were used. Cooling was brought about in 5 C. steps by immersing the sealed vials containing the semen in water baths of successively lower temperatures (20, 15, 10, 5, 0 C.). The time interval between successive 5 C. steps for each sample was held constant throughout cooling but was varied with different samples from 10 to 120 minutes as indicated below in table II. In all cases, the samples were stored at 0 C. after being cooled step-wise to this temperature.

*Glucose—phosphorus—calcium.

Comparison of Yolk-Phosfate Diluted and Normal Semen when Cooled at Different Rates.—Again the percentage of live sperm remaining in the undiluted samples was greater with the slower rates of cooling. However, when the semen was diluted 1:3 with yolk-phosfate diluter, the percentage of live sperm was not influenced by the rate of cooling. It is not known how rapidly semen diluted with yolk-phosfate diluter can be cooled and still maintain the maximum number of live sperm, but if this diluted semen is immersed immediately in a water bath at 0 C. there is a significant decrease in the percentage of live sperm.

TABLE I*.—Influence of Seven Diluters on the Percentage of Live Sperm in Fresh and Stored Bull Semen**

IN	Percentage of live sperm Hours in storage 0 hr.	24 hr.	72 hr.	pH	O.P.†
Undiluted	69.5	22.5	1.9	6.8–7.0	0.608
GPC 2	49.9	3.3	0.0	5.37	0.465
GPC 4	48.7	2.9	0.0	5.54	0.489
GPC 6	52.0	12.8	0.0	6.76	0.475
GPC 7	62.1	13.3	0.0	5.04	0.587
Sulfate	70.0	14.1	0.0	5.10	0.624
Phosfate	69.5	45.3	23.4	7.00	0.587
Tartrate	72.0	13.0	0.0	5.8	0.604
Yolk-Phosfate	76.8	74.2	69.6	6.96	0.536

*Data are the averages of four trials.
**Collected in Arizona.
†O.P. = Osmotic pressure: Depression of freezing point below that of water (C.).
GPC-2 equals anhydrous glucose, 60 Gm.; $Na_2HPO_4 \cdot 12\ H_2O$, 33.6 Gm.; KH_2PO_4, 3.2 Gm.; $CaHPO_4$, 1.0 Gm.; $MgHPO_4$, 1.0 Gm.; H_2O, 2,000 cc.
GPC-4 equals anhydrous glucose, 57 Gm.; KH_2PO_4, 1.4 Gm.; $Na_2HPO_4 \cdot 12\ H_2O$, 34.1 Gm.; $CaHPO_4$, 0.1 Gm.; $MgHPO_4$, 0.1 Gm.; Na_2SO_4, 1.7 Gm.; H_2O, 2,000 cc.
GPC-6, same as GPC-4, plus lecithin, 2.0 Gm.
GPC-7, same as GPC-6, plus salt-free peptone, 20 Gm.
Sulfate equals H_2O, 1,000 cc.; Na_2SO_4, 13.6 Gm.; anhydrous glucose, 12 Gm.; salt-free peptone, 5.0 Gm.
Phosfate equals $NaHPO_4$, 1.35 Gm.; KH_2PO_4, 0.66 Gm.; H_2O, 100 cc.
Tartrate equals H_2O, 1,000 cc.; anhydrous glucose, 12 Gm.; $KNaC_4H_4O_6$, 25.6 Gm.; salt-free peptone, 5.0 Gm.
Egg-Phosfate equals egg yolk, 100 cc.; H_2O, 100 cc.; KH_2PO_4, 0.2 Gm.; $NaHPO_4$, 0.8 Gm.

Storage Temperature and Sperm Survival.—The results presented in table 4 indicate, on the basis of percentage of live sperm and motility rating, that 5 C. was superior to any temperature tried for the storage of bull semen.

The samples (0.5 cc. in each case) were cooled to the storage temperature in 5 C. steps of 1 hour duration by immersing them in water baths of successively lower temperatures. After 24 hours storage, the samples were warmed to 35 C. and the motility observed.

DISCUSSION

In any study of diluters for mammalian semen, a distinction should be made between those which act as diluters only and those which, in addition, are capable of prolonging sperm survival during storage. The diluter which is used for dilution only might prove satisfactory for immediate insemination, yet possess characteristics that are harmful to the sperm. The ideal diluter for semen storage must possess the chemical and physical characteristics capable of establishing and maintaining a suitable environment for the sperm cell.

The percentage of live sperm existing in the semen samples (table I) immediately following dilution would indicate that some of the diluents listed may not be harmful to sperm if the diluted semen is used for immediate inseminations. However, the results obtained after 24 and 72 hours (table I) indicate that only two of the diluters are capable of prolonging sperm survival during storage. The two diluters, phosfate buffer and yolk-phosfate, possess and maintain the proper environment for the sperm as indicated by initial pH and osmotic pressure (table I) and buffering capacity (figure 1). All the other diluters studied were deficient in their capacity to maintain the pH within the physiological range of bull semen during storage (figure 1). In addition, these diluters except GPC 6 had a very low pH (table I).

Moore and Mayer,[13] and Moore, Mayer and McKenzie[12] have shown the harmful effects of low pH on sperm activity in ram semen. In every case, the GPC diluters produced definite decreases in motility and in the number of live sperm upon addition to the semen. The tartrate and sulfate diluters, however, did not produce comparable

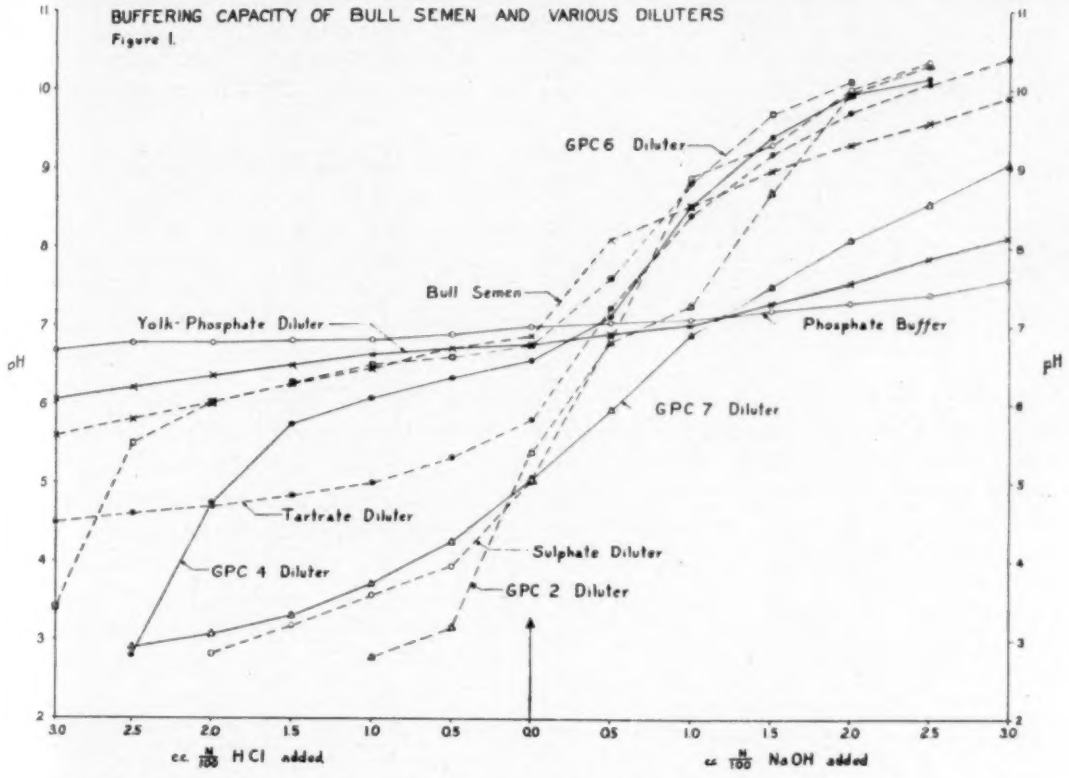

Fig. 1.—Curves showing the changes in pH of bull semen and various diluters induced by the addition of measured quantities of N/100 NaOH and HCl. (Buffering capacity).

TABLE II.—The Percentage of Live Sperm Remaining in Semen Samples* After Cooling at Different Rates

Storage time at 0 C.	Intervals between 5 C. steps in cooling Minutes					
	10	20	40	60	90	120
9 hrs.	4.4	11.3	24.8	37.8	42.5	43.0
24 hrs.	0.0	3.8	18.6	29.4	33.6	34.0

*Semen collected from bulls in Arizona, average of 3 trials.

decreases in motility and number of live sperm. This suggests their practicability for use in insemination soon after dilution.

It is suggested, then, that in every diluter used for the storage of bull semen, the pH, buffering capacity, and osmotic pressure should be well within the physiological range for these physical characteristics of normal bull semen.

Very little motility, and a low per cent of live sperm were found after 24 hours in the majority of samples of semen collected in Arizona when stored undiluted. However, in agreement with the work of Phillips and Lardy[14] and Willett, *et al.*,[21] the semen collected in Arizona consistently maintained good motility and a high per cent of live sperm in yolk-phosfate diluter.

This is contrary to the behavior of the bull semen collected in Missouri which stored undiluted satisfactorily for a period of several days. Swanson and Herman[17] working in Missouri, and Underbjerg and Davis[18] working in Nebraska have also re-

TABLE III.—The Percentage of Live Sperm in Undiluted and Yolk-Phosfate Diluted Semen* When Cooled at Different Rates

Storage time at 0 C.	Treatment of semen	Intervals between 5 C. steps in cooling Minutes			
		10	20	40	60
24 hrs.	Undiluted	33.3	38.0	40.5	51.2
	Diluted	61.3	61.5	62.2	62.6
72 hrs.	Undiluted	22.6	32.9	36.0	42.3
	Diluted	57.2	57.1	62.1	57.4
216 hrs.	Undiluted	15.6	20.8	24.7	32.4
	Diluted	56.0	57.8	60.4	56.9

*Semen collected from University of Missouri bulls, average of 2 trials.

ported the satisfactory storage of undiluted bull semen for periods of 24 hours and longer. Conversely, Phillips and Lardy[14] in Wisconsin and Willett, Fuller, and Salisbury[21] in New York indicated the necessity of the yolk-phosfate diluter for which the effects of the rate of cooling on yolk-phosfate diluted and undiluted semen were compared, rapid cooling was again shown to decrease the percentage of sperm surviving storage in undiluted semen (table III). However, as previously re-

TABLE IV.—The Percentage of Live Sperm and Motility Readings of Bull Semen* Stored at Various Temperatures

Storage time	Storage Temperatures				
	20 C.	15 C.	10 C.	5 C.	0 C.
8 hrs.	76.0	72.2	67.1	75.2	66.5
24 hrs.	42.4	25.4	46.6	63.0	50.4
Motility rating after 24 hrs.	0	1	2	3	2

*Semen collected from bulls in Arizona, average of 2 trials.

the satisfactory storage of bull semen during similar periods of time.

Undiluted bull semen is very sensitive to the effects of rapid cooling, especially at lower temperatures. The results in tables II and III show a definite relation between the rate of cooling a semen sample and the number of sperm surviving after storage. In table II, the effects of the most rapid cooling rate are strikingly illustrated by the very low percentage of sperm surviving a nine-hour storage period and the complete absence of live sperm after 24 hours of storage. The data in this table also point to the increase in the percentage of sperm surviving storage as the rate of cooling was progressively decreased. The maximal percentage of sperm survival was reached when the interval between successive 5 C. steps was 90 minutes.

In a second series of experiments, in ported by Lasley, *et al.*,[8] semen diluted with yolk-phosfate buffer not only had a higher percentage of live sperm after storage than any of the undiluted samples, but the number of live sperm remaining was the same whether the sample was cooled rapidly or slowly.

In addition to the effect of the rate of cooling, the number of sperm surviving storage is also influenced by the storage temperature. The results of an experiment, in which the rate of cooling was kept constant (table IV), indicate, on the basis of the percentage of sperm surviving and motility rating, that 5 C. was superior to the other temperatures tried for the storage of undiluted bull semen. Bacterial growth was very great in the samples which were stored at the higher temperatures (15 C. and 20 C.). Gunsalus, Salisbury and Willett[5] state that the storage temperature

must be 5 C. or lower to prevent rapid bacterial growth. The slower growth of bacteria at 5 C. may be a factor favorably influencing the storage of undiluted semen at this temperature.

Semen diluted with yolk-phosfate apparently is not influenced to the same degree as undiluted semen by the temperature at which it is stored.

CONCLUSIONS

In the preparation of semen diluters, the physical and chemical characteristics should be given careful consideration, particularly if the diluted semen is to be stored.

Both phosfate buffer and yolk-phosfate diluter possessed and maintained the physical characteristics—pH, osmotic pressure, and buffering capacity—adequate for the proper storage of bull semen.

Only the yolk-phosfate diluter protected the sperm from the detrimental effects of rapid cooling. In addition, the influence of storage temperature upon sperm survival was definitely reduced by dilution of bull semen with yolk-phosfate.

In the storage of undiluted bull semen, a definite relationship exists between the rate of cooling and sperm survival. The rate of cooling most satisfactory for undiluted semen was that of 5 C. steps at 90 minute intervals.

On the basis of motility and sperm survival, 5 C. proved to be the best temperature for the storage of undiluted bull semen.

Evidence from the results of the present investigation and those of other investigators has been cited indicating differences in the storage potentialities of bull semen studied in different localities.

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The Normal Cerebrospinal Fluid Pressure and a Method for Its Determination in Cattle

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DURING THE COURSE of some experimental work in which the relationship of vitamin A deficiency to cerebrospinal fluid pressure was being investigated (Moore and Sykes[1]), it became necessary to develop a method for determining the pressure of this fluid in cattle. The method adopted and the values obtained with this procedure are described below.

The recording of spinal fluid pressures in this work was first attempted by lumbar puncture. However, in spite of the fact that no difficulty was experienced in puncturing the spinal canal, it has so far proved impossible to obtain spinal fluid by penetrating this region. This is probably due to the fact that the spinal cord extends much farther caudally in cattle than it does in the human subject. Puncture into the subarachnoid space was then attempted through the dorsal opening of the atlanto-occipital articulation and in this location no difficulty has been experienced in obtaining spinal fluid.

It early became evident that the animals must be kept in the standing position to obtain normal pressure values. When the animals were cast, pressures exceeding 300 mm. of saline were recorded. The pressure of these same animals in the standing position was approximately 100 mm., and in view of the reported values for other species (1 to 7), these seemed more nearly correct. The pressure produced by the abdominal contents was responsible for the high spinal fluid pressures which were recorded when the animals were lying on the side. A similarly high spinal fluid pressure can be produced with the animals in the standing position by increasing manually the pressure within the abdominal cavity.

The details of the method finally adopted for determining the pressure of the cerebrospinal fluid are as follows:

Restraint is applied by two attendants. One attendant faces the animal and draws the head tightly toward his abdomen and chest with his arms over the horns, if present, and his hands locked under the jaw. During insertion of the needle the head is flexed slightly, but once the puncture has been made it is held in the normal position. The head is held as steady as possible. The second attendant applies whatever further restraint is necessary. Every precaution must be taken to see that the animal is standing naturally, is offering no resistance to the attendants, and is not visibly excited when the record is taken.

No anesthetic is necessary in making the determination. Local anesthesia usually causes more excitement and resistance than the procedure itself. With a little training, calves submit to this procedure without undue resistance in most cases, and generally, once the puncture has been made, they stand quietly with no visible signs of excitement. Restraint is more difficult in mature animals, however. If the animals have not become accustomed to the procedure during their development, this method is not recommended unless they are unusually docile.

Sixteen-gauge hypodermic needles with a short bevel are used. In young animals, a 75 mm. needle is sufficiently long, but in larger calves and mature cows, a 100 mm. needle must be used. In instances where a considerable crest has developed, the 100 mm. needle may not be of sufficient length, and obviously no determinations can be made unless the pressure exceeds normal values and a longer needle is employed. A

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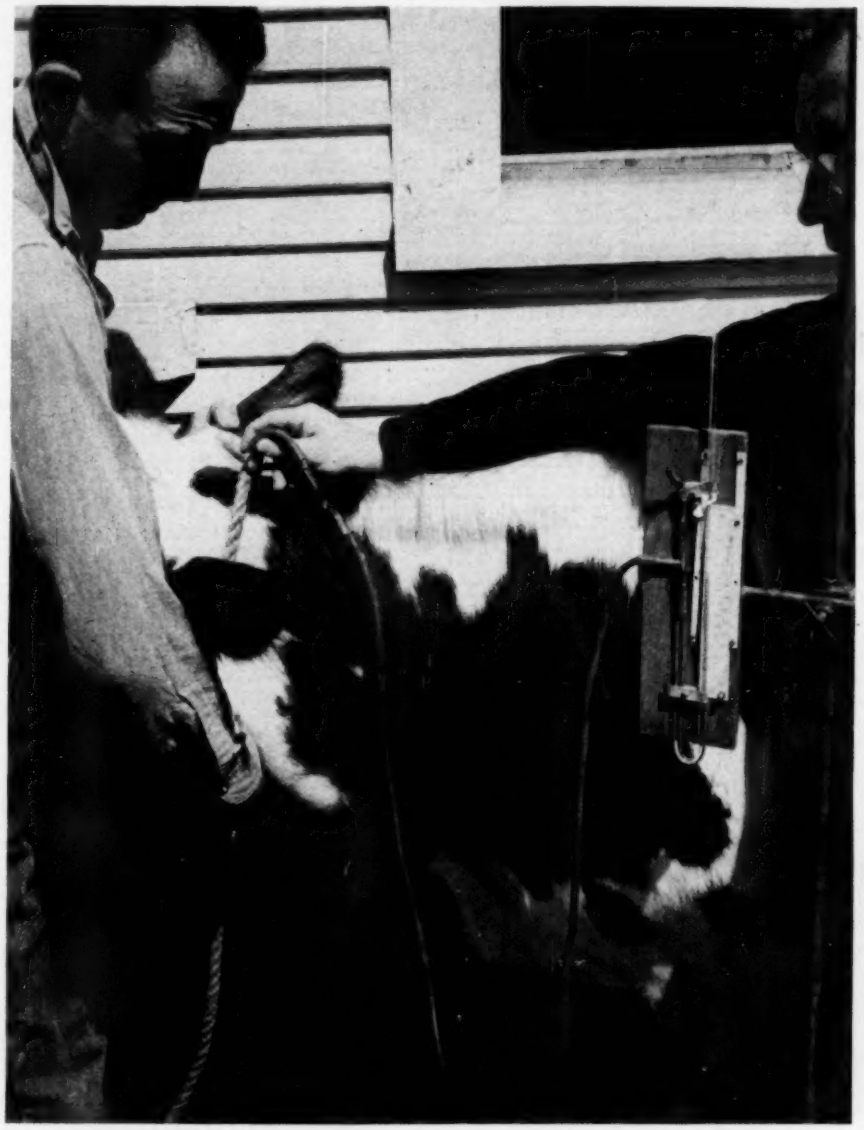

—After Sykes and Moore, 1942

Fig. 1.—Demonstrating the use of the manometer in determining the cerebrospinal fluid pressure in a Holstein-Friesian calf.

stylet of the exact length and gauge of the needle is left in the needle during the insertion to prevent blockage and escape of fluid.

Insertion is made through the dorsal opening of the atlanto-occipital articulation. The point of insertion through the skin and underlying tissues is located on the midline, 2 to 3 inches posterior to the attachment of the ligamentum nuchae and in a line with anterior prominences of the atlas. When the point of the needle has reached the level of the vertebral column, the opening is found by moving the point backward and forward. If the penetration is made as outlined, the opening will be directly below it. The needle is gently passed through the opening in the articulation. Its emergence is signaled by a freer passage of the needle. Extreme care now must

be exercised for the puncture of the dura and arachnoid coats, in order to prevent damage to the spinal cord. A rather quick jab, traveling only 1 or 2 mm., is to be preferred to a slow, steady push. It is important that the point of entrance be on the median line. If the point of the needle becomes deflected to one side or the other, the puncture causes hemorrhage which makes a measurement of pressure impossible until the bleeding has ceased.

When the puncture has been made, the stylet is withdrawn and the needle connected by rubber tubing to a manometer of 2 mm. bore. The tubing and manometer are completely filled before attachment with 0.85 per cent saline, which is retained by a pinch cock on the rubber tubing. The side arm of the manometer is set at approximately the same leved as the hub of the needle, the free end of the connecting tube held unattached on a level with the hub of the needle, and the pinch cock released. When the saline in the manometer reaches a constant level, the zero point of a sliding scale is adjusted to this level and attachment to the needle is then made. The pressure recorded on the manometer, plus the length of the needle, is the spinal fluid pressure. The manometer is left attached to the needle long enough for any excitement effects which may not be noticeable externally to subside, and the lowest constant value is recorded. After the record is obtained, the tube is detached and the zero level checked. Fig. 1 shows the arrangement of the apparatus.

While records obtained in this way may not be absolutely accurate since it is practically impossible to immobilize the head completely, it is believed that they are accurate to within a few millimeters. The values obtained in normal calves do not show marked variations in the same animal, nor from animal to animal, and are within the normal limits as given for the human subject and the dog (100 to 130 mm. of saline). The values shown in table I, which were obtained on three animals, are typical. These values were obtained at 1- or 2-week intervals.

TABLE I.—Cerebrospinal Fluid in Calves Taken at Frequent Intervals

—Animal 411—		—Animal 432—		—Animal 430—	
Days old	P. S.	Days old	P. S.	Days old	P. S.
492	100	86	115	48	93
502	110	93	90	62	90
516	103	101	110	89	95
531	87	108	100	103	103
		115	95	121	105
		126	110	123	110
		140	105		

P. S.=Pressure mm. saline.

Normal Cerebrospinal Fluid Pressure of Cattle

Using the method outlined above, pressures have been obtained on 21 cattle ranging in age from 30 to 700 days. Successive records over considerable periods have been recorded for many of these individuals. The pressures recorded at various age levels are shown in table II.

TABLE II.—Cerebrospinal Fluid Pressures in Cattle at Various Age Levels

Age <100 days	Age 100-200 days	Age 200-300 days	Age 300-500 days	Age 500-700 days
(mm. Saline)	(mm. Saline)	(mm. Saline)	(mm. Saline)	(mm. Saline)
115	120, 120	80	110	97
93	100, 110	90	120	120
135	150, 108	100	97	127
107	95, 95	120	100	90
85	125, 110	130	115	110
93	105, 105	100	100	110
90	75, 130	150	...	103
95	85, 100	90	...	125
125	85, 100	95	...	107
100	80, 103	110	...	122
90	95, 105	85	...	110
...	90, 110	...	...	103
...	105, 115	...	...	87
...	120, 105	...	...	...
...	90, 120	...	...	...
...	105, 85	...	...	...
Avg. 102.6	107.7	104.6	107.0	108.5

The average cerebrospinal fluid pressure for all the determinations was 105.1 mm. of saline. The minimum value obtained was 80 mm. and the maximum 150 mm. (2 instances).

Table III illustrates the relative constancy of the cerebrospinal fluid pressure

in individual animals during at least the first year of development.

TABLE III.—Cerebrospinal Pressure Values During the First Year of Development

Animal 437		Animal 441		Animal 456		Animal 457	
Days old	P.S.	Days old	P.S.	Days old	P.S.	Days old	P.S.
49	125	115	120	111	80	107	75
63	100	156	105	146	95	142	85
124	120	189	85	181	90	177	85
165	100	202	90	208	110	204	90
200	130	223	100	260	85	256	95
234	100	285	120	296	90	292	90
267	150	304	110	319	105	315	120
		359	120	345	105	341	95
				402	90	398	90

P. S.=Pressure mm. saline.

Discussion of Results

While it would seem desirable to have some good method for checking the accuracy of the method described in the first section of the paper, it is thought that the values reported are at least reasonably close to the actual cerebrospinal fluid pressure of cattle. A few observations made with other types of manometers always gave good checks, and the constancy of the values shown in tables I and III certainly indicates that results are reproducible by this method and would also suggest that the method is capable of recording the actual pressure. Data on larger numbers are needed, of course, before these conclusions can be considered exact.

Our experience with puppies has suggested that the cerebrospinal fluid pressure in young calves might be somewhat lower than that of more mature animals. For that reason the data in table II were arranged as shown. The data presented there certainly offer no suggestion that the cerebrospinal pressure varies with age. The data in table III likewise show that the pressure in individual animals remains constant at least up to one year of age, and comparison of the data with the pressures recorded for the 500- to 700-day group (table II) suggests that it remains constant practically until maturity.

Summary

A method for measuring the cerebrospinal fluid pressure in the bovine species is described.

Using this method, it was found that the average pressure in cattle up to two years of age was 105.1 mm. of saline. The minimum value observed was 80 mm. and the maximum 150 mm.

The data suggest that the cerebrospinal fluid pressure of cattle does not vary with age, at least for the first two years of life.

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An Anomaly in Hog Hearts: Defectus Interventricularis Septi

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CONGENITAL anomalies of the heart, as far as my observations extend, appear to be prevalent in hogs and include various conditions. Some of the defects I have observed are: patent foramen ovale, patent ductus arteriosus, abnormalities of the pulmonary and aortic valves in the form of two leaflets, fenestration of the leaflets in the part between the free edge and the line of closure, fibrous cords stretching from one part of the surface to another in the ventricles, deficiencies of the interventricular septum, and aneurysms of the aortic and pulmonary arteries.

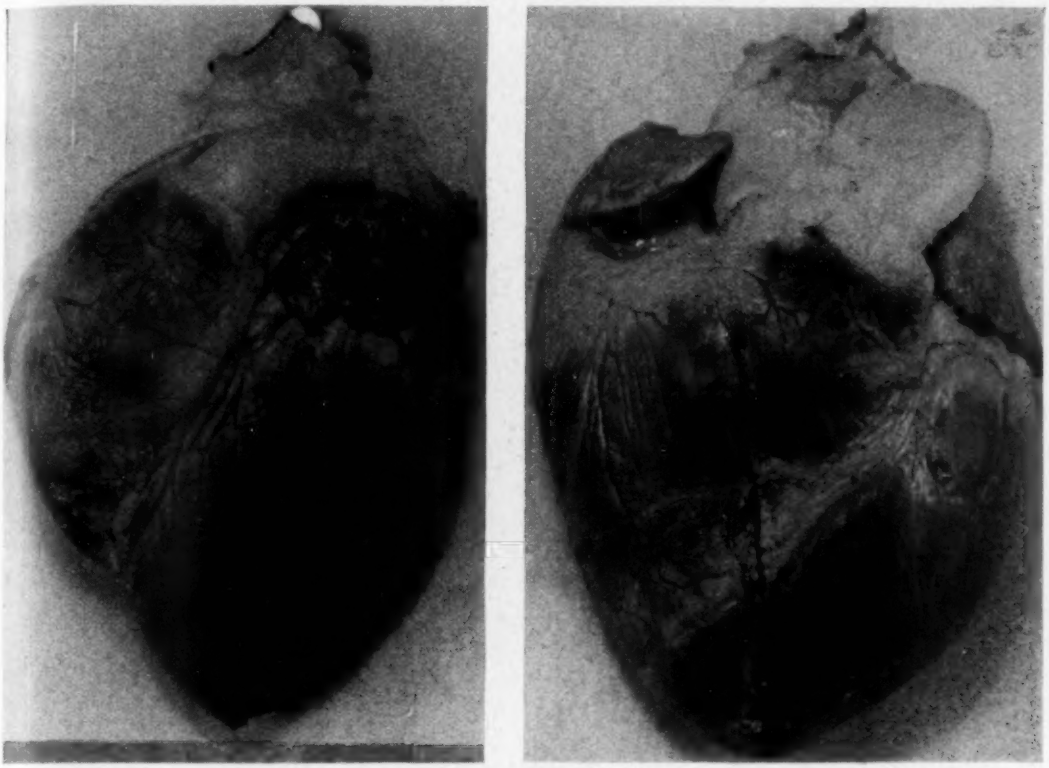
The following is a discussion of patencies of the interventricular septum in hog hearts. The cases hereinafter described, to which have been assigned numbers, are considered typical and were encountered during routine postmortem meat inspection. It would be well to make clear at the beginning that all hogs affected with the patency of the interventricular septum were in apparently good condition; however, it was evident that the reserve power of these hearts is lessened.

Hog 1.—The heart from this animal, a 250-lb. sow, is pictured in the accompanying photographs and a normal heart is used for comparison. It shows the left ventricle greatly hypertrophied and dilated, especially at its base. The apex of the heart was blunt. The caliber of the aorta, as well as of the pulmonary artery, was about twice as large as normal. The aorta was affected from the sinus arteriosus to the region of the ligamentum arteriosum and the pulmonary artery was affected from the conus arteriosus to the ligamentum arteriosum. Probably because of the spiral arrangement of the cardiac muscle, the entire heart was hypertrophied, the greater effects being evidenced on the part receiving the greater stress of work. In this case, it was the left ventricle, for there was apparently a venous arterial shunt. The foramen permitted the escape of some of the venous blood directly into the arterial blood of the left ventricle, producing great pressure in the region of the aortic valve. Hence, the left ventricle was greatly hypertrophied over its entire extent, and more so at the base, as prominently evidenced externally by a bulge in the region of the coronary groove. It is apparent that the papillary muscles of the left ventricle are greatly hypertrophied and the chordae tendinae are markedly lengthened and thickened. It is also evident that the mitral valve and aortic cusps are abnormally dilated and thickened. In addition, the sinuses of Valsalva are quite capacious, the pulmonary valve is enlarged and thickened, and its sinuses are correspondingly dilated. Patency of the interventricular septum has been seen on numerous occasions in hogs and some representative cases will be described.

Hog 2.—In this animal, a hog about a year old, the heart was slightly hypertrophied and dilated, especially on the left side. These were the first abnormalities that attracted my attention. An interventricular septum patency was directly below the aortic valve on the left side and was about ⅜ inch in diameter. On the right side of the interventricular septum, the foramen was about ¼ inch in diameter and was covered by a slightly malformed right tricuspid valve. An unusually thin moderator band was found attached in the region of the patency of the interventricular septum. Two cusps, slightly fenestrated, made up the aortic valve, and the valve itself was markedly thickened.

Hog 3.—In a hog about a year old there was an extensive dilatation of both auricles associated with a hypertrophy of both ventricles. The interventricular septum

From the United States Bureau of Animal Industry, Chicago.



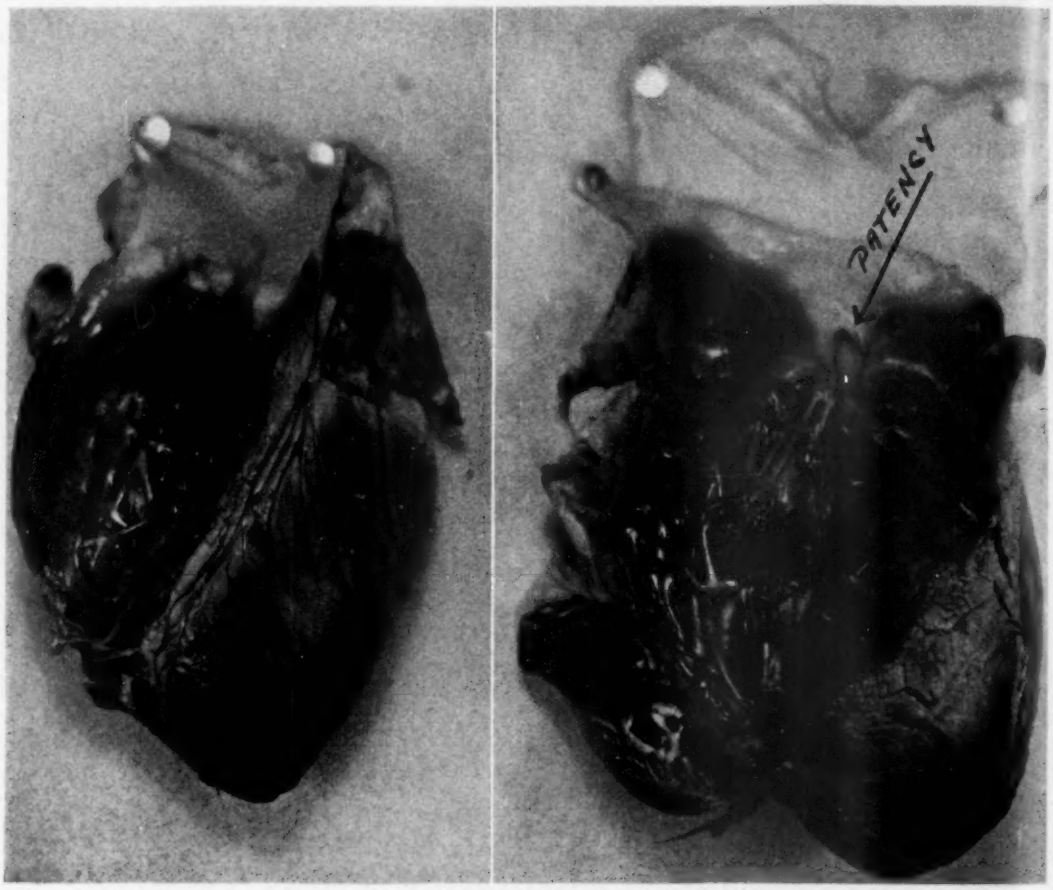
Hog 1—Exterior view. Left heart, normal; right heart—hypertrophic due to interventricular septum patency.

foramen was below the aortic valve on the left side of the septum and below the pulmonary valve on the right. There was a diffuse aneurysm of the aorta which extended from the sinus arteriosus to the ligamentum arteriosum. The aneurysm was associated with an extensive sclerosis of the aortic and mitral valves. This heart anomaly was found during examination of the hog, which was retained and condemned for a septicemia.

Hog 4.—This hog was about a year old. The interventricular septum of the heart presented quite a large patency, fully ¼ inch in diameter, below the aortic valve. In addition, there was an open foramen ovale. The entire heart was dilated and hypertrophied; however, the change in size was more marked in the right auricle and right ventricle. The point of the right ventricle, being level and on an even plane with the point of the left ventricle, produced a square-shaped apex. Both ventricles showed moderator bands which were hypertrophied. The papillary muscles of the right ventricle were markedly hypertrophied. An aneurysm was found at the origin of the pulmonary artery. The defect of the interventricular septum in this case was below the aortic and pulmonary valves. There was a sclerosis of the aortic, pulmonary and right tricuspid valves. This heart presented evidence of an arterial-venous shunt of blood.

Hog 5.—In this hog, a young animal, there was a globular-shaped heart associated with a very slight foramen of the interventricular septum. This patency was about ⅛ inch in diameter.

Hog 6.—In a young hog, the left ventricle of the heart was hypertrophied and bulged in the region of the coronary groove. The apex was rounded and there was a diffuse aneurysm at the origin of the pulmonary artery, extending from a prominent and enlarged conus arteriosus to the ligamen-



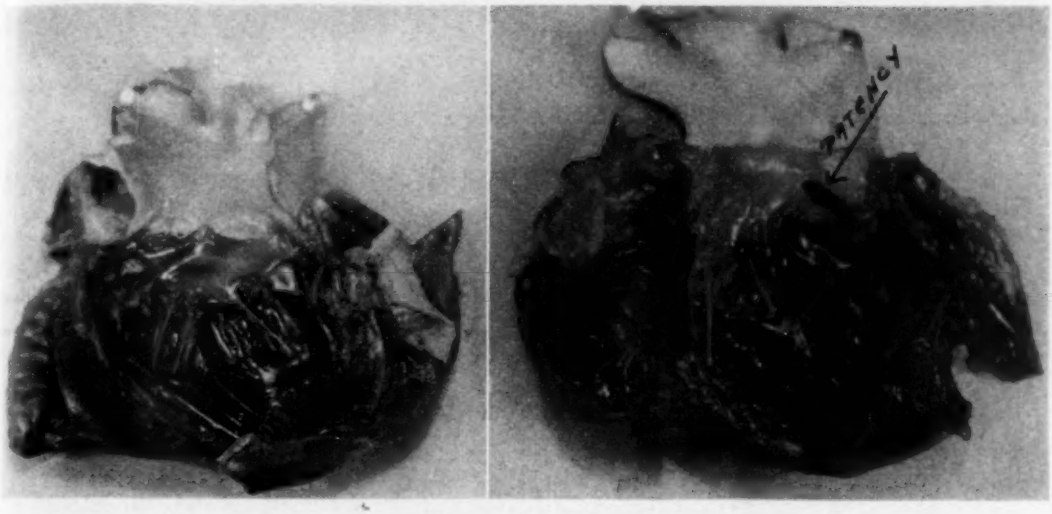
Hog 1—Interior of left ventricle exposed. Heart to left, normal; heart to right—arrow points to interventricular patency.

tum arteriosum. All the changes in the heart associated with this abnormality of the interventricular septum were probably due to an arterio-venous shunt of the circulation.

Hog 7.—In a young hog, there was an extensive diffuse aneurysm of the origin of the pulmonary artery, extending from the conus arteriosus to the ligamentum arteriosum. The right ventricle was hypertrophied and enlarged. A foramen was found in the interventricular septum. Two cusps made up the pulmonary valve; that is, the two posterior cusps were united into one large cusp. The moderator band was placed high up in the right ventricle and was unusually short and thick. There was a slight fibrosis of the epicardial surface of the apex. The apex presented a square-shaped appearance, probably due to the fact that the right ventricle extended as far down as the left ventricle.

Hog 8.—In a young hog, there was cardiac hypertrophy; that is, the heart was about four times its normal size. There was an extensive dilatation of both auricles and an unusually large patency of the interventricular septum below the aortic valve. The foramen was about one inch in diameter.

Hog 9.—In a young hog, there was a patency of the interventricular septum of the heart which was ½ inch in diameter and below the aortic valve on the left side. The foramen opened in the region of the tricuspid valve on the right side. In this hog there were dilatation of the pulmonary artery at its origin and fibrosis



Hog 1—Interior of right ventricle exposed. Heart to left, normal; heart to right—arrow points to interventricular foramen.

of the pulmonary valve. The fibrosis extended into and markedly involved the ventricular wall. In association with a thickening of the right tricuspid valve, there were dilatation and hypertrophy of the right auricle and right ventricle. The foramen ovale had not completely closed, but left a slight patency.

Hog 10.—In a young hog, the heart was slightly enlarged. The left ventricle was especially hypertrophied and dilated, and its apex was square. There was a patent foramen ovale and an open interventricular septum that was very large, being about ¾ inch in diameter. The foramen was below the aorta on the left side of the interventricular septum and was covered by the right tricuspid valve on the right side of the septum. The pulmonary artery was extensively dilated and the pulmonary valve was markedly stenotic, being thickened, malformed and fibrotic, with two of the sinuses contracted. The right tricuspid valve was malformed and thickened. The right auricle was slightly elongated and there was a slight fibrosis on the epicardium of the apex.

Hog 11.—In a young hog, there was an extensive diffuse aneurysm of the origin of the pulmonary artery, extending from the conus arteriosus to the ligamentum arteriosum. This aneurysm was associated with an open interventricular septum which was about an inch below its usual place on the left side of the septum. There was a thick moderator band. The right ventricle was dilated and hypertrophied. The aortic valve showed fibrous strands extending to and adhering to the region of the patency, causing tension near the coronary artery.

Hog 12.—In a young hog, the interventricular septum presented a small opening which was about ⅛ inch in diameter below the aortic valve on the left side of the septum. The foramen ovale showed two small patencies near the line of closure. Both pulmonary artery and aorta were larger in caliber than normal at their origins. The heart was apparently normal in size.

Hog 13.—In a young hog, the left ventricle was dilated and hypertrophied and the apex was square-shaped. There was an unclosed foramen ovale and the right auricle was enlarged and dilated. There was a small patency of the interventricular septum to the left of the moderator band on the right side of the heart, and another on the right side of the heart which was larger and in the region of the tricuspid valve. Both communicated with a foramen of the interventricular septum opening into the left ventricle. This flaw was below the aorta and quite large. The mod-

erator band was hypertrophied and the right tricuspid valve was malformed. The volume of the right ventricle was diminished because of the intrusion of the left ventricle, which pushed the septum to the right because of its dilatation and hypertrophy. The heart was not significantly enlarged.

Hog 14.—In a young hog, the interventricular septum contained an opening which was about ⅜ inch in diameter. It was directly below the aortic valve on the left side and about an inch below the pulmonary artery on the right. One of the aortic cusps was stretched and dilated and dipped down into the patency; in fact, almost protruding into the right ventricle. There was a thin line of fibrosis around the conus arteriosus. The pulmonary artery showed two cusps which were extensively sclerosed. In addition, the origin of the pulmonary artery showed a diffuse aneurysm, as did the origin of the aorta. Each aneurysm extended to the ligamentum arteriosum, the former originating at the conus arteriosus and the latter at the sinus arteriosus. There was some fibrosis in the region of the left ventricle below the aortic cusp. The right ventricle was enlarged and its apex was square, extending as far down as the apex of the left ventricle. The right auricle was elongated. The coronary artery which extended down on the surface of the heart toward the apex appeared to be thickened and sclerosed.

According to Levine and Abbott, the following conclusions appear to be significant for patency of the interventricular septum when it occurs in man. In man, patency of the interventricular septum is quite common and ranks next in frequency to auricular defects. Foramen of the interventricular septum is most commonly associated with other abnormalities of the heart but may occur as an only lesion.[1] This condition is localized at the base of the heart just anterior to the pars membranacea and opens on the side of the right ventricle behind the septal tricuspid segment; less commonly it lies more anterior and opens into the conus of the right ventricle (bulbar septal defect), or in a few rare cases it may lie in the lower part of the septum.[2] Fibrosis on the inner wall of the ventricles directly opposite the patency of the interventricular septum indicates constant jets of blood striking that part as it comes from the opposite ventricle. Dilatation and hypertrophy accompanying this condition may be present to a greater or lesser degree, depending upon the size of the opening and the amount of blood shunted to either side. If the volume of blood is sufficiently large, it may cause enlargement of the pulmonary artery when the strain is on the left ventricle. Very large defects often lead to considerable cardiac enlargement, but they are often complicated by other conditions. Congenital defects may result in insufficiencies, venous-arterial shunts, arterial-venous shunts, etc. My observations lead me to believe that the conclusions as described above for man also hold true for hogs.

[1]Levine, Samuel Albert: Clinical Heart Disease. W. B. Saunders Co., Philadelphia (1937).

[2]Abbott, Maude E. Seymour: Atlas of Congenital Heart Disease. American Heart Association, New York (1936).

SUMMARY

1. Fourteen hogs, presenting patency of the interventricular septum as found on routine postmortem meat inspection, are discussed.

2. All the animals were young, and apparently the anomaly did not affect their physical condition.

3. The writer's observations appear to indicate that congenital anomalies are prevalent in hogs.

4. The same conclusions described in man for patency of the interventricular septum also hold true for the condition of the hog.

Studies of the Hemoglobin Content of Chicken Blood and Evaluation of Methods for Its Determination

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METHODS for the determination of hemoglobin in mammalian blood have been extensively investigated, but the literature contains only meager data concerning its estimation in the blood of the domestic fowl, as is shown in table I. In this table, to facilitate comparisons of the hemoglobin values reported by different investigators, all of the values which were presented in per cent have been converted into grams of hemoglobin per 100 cc. of blood.[13] From these data, it is seen that values, ranging from 6.56 Gm. to as high as 18.6 Gm. of hemoglobin per 100 cc. of blood, have been reported. Some of this variation may be due to differences in age, sex, and the environment of the chickens used. However, a comparison of the values given for hemoglobin in the adult male shows that the still-existing variation may be influenced by the methods employed.

In view of these diverse results of attempts to apply to chickens the methods used for determining hemoglobin in mammalian blood, it seemed advisable to make further studies and to compare several of the methods which are in common use. Some of the objectives of these studies are: 1) to determine which, if any, of the existing methods are adaptable to the chicken; 2) to find an explanation for the failure of the methods which are considered as most reliable for mammalian blood to give equally reliable results with chicken blood; 3) to develop modifications of existing methods or to devise different and practical procedures by which the hemoglobin content of chicken blood may be accurately determined; and 4) to establish standards for normal hemoglobin values of chicken blood.

A careful review of the literature revealed that much of the difficulty in the determination of hemoglobin in chicken blood was probably related to the peculiarities of constitution of chicken blood cells and of the behavior of the blood elements when subjected to the procedures for hemoglobin determination of mammalian blood. The more significant of these will be briefly described.

One of the difficulties encountered in determining the hemoglobin content of chicken blood by the Van Slyke method is the ropy, slimy gel which forms upon hemolysis. Schultze and Elvehjem,[52] Klein, Hall and King[31] concluded that the method did not give consistent results because of incomplete hemolysis and the trapping of oxygen by the gel. Another disadvantage of the Van Slyke method is the time involved in making the test.

The determination of hemoglobin from the iron content of a blood sample gives inaccurate results with chicken blood because of the nonhemoglobin iron in the red blood cells, as was shown by Barkan[2] and Wingarden and Borsook.[57] The former reported the amount of such iron to be from 4 to 6 per cent of the total and the latter, using 2 chickens, 5.3 and 5.4 per cent, respectively.

The colorimetric-acid-hematin methods consist essentially of converting the hemoglobin of a blood sample to acid hematin with a mineral acid and comparing the color with a standard. Although, ordinarily, the solution of acid hematin prepared from mammalian blood by this method appears clear, Terrill[55] found that turbidities did occur and that their extent was not proportional to the amount of hemoglobin present. Avian blood presents a far greater problem since the turbidity is much more pronounced, making color matching extremely difficult and the resulting hemoglobin values too high. Dukes and Schwarte[10] report that

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the suspended substance is probably caused by the abundance of nuclear material. In an attempt to compensate for the high hemoglobin values, they introduced a correction factor for the presence of the turbidity, but whether the factor is elastic enough to compensate for the wide variabilities that occur has not been shown.

Schultze and Elvehjem[52] devised a modification of Newcomer's method for use with chicken blood in which a clear solution of acid hematin was produced by first laking the blood in a dilute solution of ammonia before acidulating with hydrochloric acid.

The production of a stable and clear standard is one of the greatest problems in any colorimetric method of determining hemoglobin. Since the introduction, in 1878, of picrocarmine for this purpose, many standards have been proposed for matching the color of acid hematin or some other hemoglobin derivative prepared from whole blood.[3, 6, 14, 24, 42, 43] Some of these are prepared as solutions and others are colored glass, but both types have certain disadvantages. Objections to the use of liquid standards for hemoglobin determinations are the amount of work required in their preparation and the necessity of constant checking for deterioration of the solutions. This is especially true of those prepared from blood itself. Another disadvantage is that the artificial standards do not have the same light absorption throughout the visible spectrum as hemoglobin or the derivatives prepared from blood.

The Dare hemoglobinometer[9] is one of the oldest instruments which employs a colored glass standard, but more satisfactory methods have since been developed.

Newcomer[39] obtained a high transmission yellow glass of 0.96 mm. thickness with nearly the same absorption zone in the visual portion of the spectrum as acid hematin. Although the merits of this standard are recognized by most investigators, some disadvantages have been cited. Osgood and Haskins[42] and Robscheit[45] pointed out that the paleness of the yellow color of the Newcomer disc made it difficult to distinguish with certainty small differences in the acid hematin solutions. Thicker glass (1.02 mm.) was found even more unsatisfactory, and for this reason they favored the liquid standards.

Another disadvantage in the Newcomer standard is the variability in the yellow glass discs. Elvehjem[12] found differences of as much as 12 per cent in determinations of hemoglobin with various discs. To avoid this error, he advised that each disc be standardized with acid hematin solutions of known hemin and iron content.

Keilin[30] found that acid or alkaline hematin solutions prepared from either hemin or hemoglobin had the same absorption spectrum. This made it possible to use pure hematin solutions for standards in hemoglobin determinations.

A more recent development in the quantitative measurement of hemoglobin in diluted whole blood or its derivatives is the use of a light-sensitive cell. One such instrument (and the one used in the studies reported here) is the Sheard-Sanford photelometer.[47, 48, 54] Hemoglobin determinations with this instrument are based upon the measurement of current produced by the emergent light from a constant source on a photronic cell after the light has passed through a diluted sample of blood, or one of its derivatives, and a selective absorption filter. Sanford and Sheard report a percentage of error not to exceed 4 per cent, and averaging less than 2 per cent, when hemoglobin determinations by their method are compared with those made by the Van Slyke method. This degree of error is less, both in average amount and in limits of variability, than visual methods since accuracy of such comparisons is so greatly influenced by the sensitivity to color of the eyes of different individuals. Mellon,[36] in his discussion on the status of colorimetry, states that a lower percentage error may be expected with a well-designed photoelectric instrument than with a visual colorimeter, due to the greater sensitivity of the former.

EXPERIMENTAL CHICKENS

All birds in this experiment were single comb White Leghorn pullets, from 6 to

8 weeks of age. A constant supply of birds of this age was maintained by obtaining day-old chicks every six weeks. These were reared in battery brooders and fed an all-mash ration, which, through several years of usage, had been found to provide ample nourishment.

BLEEDING TECHNIQUE

When blood was needed for determinations, 5 or 10 chicks were brought to the laboratory. After being bled, they were placed in an isolation unit until the next bleeding date. The same birds were bled at intervals of not less than three days until the age limit of 56 days was reached. The blood was obtained by making a puncture in the wing vein, large enough to insure a free flow. The first few drops were discarded. Duplicate samples for all methods were taken from each bird at each bleeding.

METHODS FOR DETERMINATION OF HEMOGLOBIN

Comparison was made of six methods for the determination of hemoglobin. They were the Dare,[9] Haden-Hauser,[20, 21] Newcomer,[39] as modified by Schultz and Elvehjem,[52] Sanford-Sheard,[47] and the acid and

TABLE 1.—Hemoglobin Content of Chicken Blood as Reported by Various Observers

HEMOGLOBIN—PER CENT OR GM. PER 100 CC. OF BLOOD

OBSERVER	YEAR	AVERAGES AS REPORTED	RANGE AS REPORTED	AVERAGE HB IN GMS. PER 100 ML.	METHOD	NUMBER DETER.	NUMBER BIRDS	AGE SEX
Muller[33]	1886	16.75 Gm.	15.0—18.0%	16.75	Spectro	Norm.*	....**	.....
Fllerman and Bang[11]	1908	58.0%	50.0—65.0%	10.03	Sahli	Norm.	.....	.....
Hirschfeld[28]	1909	60.0%	.....	10.38	Sahli	Norm.	.....	.....
Schmeisser[51]	1915	65.0%	60.0—70.0%	11.25	Sahli	Norm.	.....	.....
Burnett[5]	1917	76.0%	48.0—85.0%	.....	.....	Norm.	.....	.....
Mack[35]	1917	87.3%	.....	.....	.....	Norm.	.....	.....
Fritsch[17]	1920	12.3 Gm.	.....	12.3	Hufners	10	10	Ad. M.
		9.6 Gm.	.....	9.6		10	10	Ad. F.
Klieneberger and Carl[32]	1927	62.0%	54.0—85.0%	10.73	Sahli	Norm.	.....	.....
Hayden[26]	1927-28	74.0%	70.0—80.0%	.....	.....	10	10	Ad. F.
Forkner[16]	1929	62.9%	46.0—77.0%	10.64	Newcomer	29	11	Ad. M.
Bayon[1]	1930	60.0%	.....	10.38	Sahli	Norm.	.....	Ad.
Hart and Others[22]	1930	.....	6.0—7.0 Gm.	.....	Newcomer	Norm.	.....	Chix
Dukes and Schwarte[10]	1931	9.8 ± .1 Gm.	.....	9.8 ± .1	Improved	304	304	Ad. F.
		8.9 ± .7 Gm.	.....	8.9 ± .7	Newcomer	101	101	Yo. F.
		13.5 ± .2 Gm.	.....	13.5 ± .2	Method	26	26	Ad. M.
Girisch[18]	1931	.....	97.5—107.5%	16.9—18.6	Sahli	Norm.	.....	Ad. M.
		.....	95.5—105.5%	16.5—18.3		Norm.	.....	Ad. F.
Romer[46]	1931	75.0%	.....	12.98	Sahli	Norm.	.....	.....
Thomsen and Engelbreth-Holm[56]	1931	50.0%	.....	8.65	Sahli	Norm.	.....	Ad.
Fenstermacher[15]	1932	76.0%	63.0—90.0%	12.16	Dare	120	12	.....
Harmon, Ogden and Cook[23]	1934	41.0%	.....	6.56	Dare	7	7	Ad. M.
Cook and Harmon[8]	1933	12.4 ± .34 Gm.	9.8—14.1 Gm.	12.4 ± .34		5	5	Ad. Cap
		14.3 ± .29 Gm.	10.6—16.8 Gm.	14.3 ± .29		11	11	Ad. F.
		8.2 ± .12 Gm.	4.0—12.2 Gm.	8.2 ± .12	Dare	64	64	Ch. F.
		7.5 ± .10 Gm.	4.4—10.2 Gm.	7.5 ± .10		69	69	Ch. M.
		11.0 ± .06 Gm.	5.3—19.0 Gm.	11.0 ± .06		606	606	Ad.
Holmes, Pigott and Campbell[27]	1933	9.7 Gm.	.....	9.7	Modified	Norm.	Norm.	42 Da. M.
		9.6 Gm.	.....	9.6	Sahli	Norm.	Norm.	42 Da. F.
		10.1 Gm.	.....	10.1		Norm.	Norm.	84 Da. F.
		9.7 Gm.	.....	9.7		Norm.	Norm.	84 Da. F.
Cook and Dearstyne[7]	1934	.....	14.75—15.5 Gm.	.....	Newcomer	5	5	Ad. M.
			7.0 —15.0 Gm.		Newcomer	75	75	Ad. F.
Gonzaga[19]	1935	9.34	.....	9.34	Wong	.....	.....	6 Wk.
		10.80	.....	10.80		.....	.....	2 Mo.

**No data.
*Number of chickens not reported. The values given represent the observer's conclusion that the number of chickens used constituted a suitable sample.

alkaline hematin photelometric methods which are discussed in this paper.

The Dare instrument was of recent manufacture. The disc was calibrated on the basis of 16 Gm. of hemoglobin per 100 cc. of blood. The same capillary cell was used throughout the experiments.[25]

The dilutions for hemoglobin determinations with the Haden-Hauser instrument were made with a 1 : 10 Thoma white cell or Trenner pipette. Infrequently, a 1 : 20 dilution was necessary for those individuals having a high hemoglobin value.

For the modified Newcomer method, 1 to 251 dilution pipettes of 5 cc. capacity were used. The concentrated hydrochloric acid

TABLE II.—Conversion of Photelometric Readings of Acid Hematin Into Gm. of Hemoglobin per 100 cc. of Blood

*PHL	**HB	*PHL	**HB	*PHL	**HB	*PHL	**HB
40.0	14.24	45.5	11.82	51.0	9.68	56.5	7.81
40.5	14.00	46.0	11.63	51.5	9.52	57.0	7.64
41.0	13.77	46.5	11.42	52.0	9.30	57.5	7.50
41.5	13.51	47.0	11.16	52.5	9.11	58.0	7.36
42.0	13.34	47.5	11.01	53.0	8.95	58.5	7.24
42.5	13.10	48.0	10.80	53.5	8.78	59.0	7.12
43.0	12.86	48.5	10.63	54.0	8.59	59.5	6.88
43.5	12.67	49.0	10.44	54.5	8.45	60.0	6.69
44.0	12.49	49.5	10.25	55.0	8.26	60.5	6.55
44.5	12.27	50.0	10.04	55.5	8.14	61.0	6.41
45.0	12.06	50.5	9.83	56.0	7.95	61.5	6.22

*Phl—Photelometer readings.
**Hb—Grams of hemoglobin per 100 cc. of blood.

was added to the laked blood sample from a microburette. The color intensity of the samples was read in from two to four hours after the addition of acid in a Bausch and Lomb Duboscq colorimeter with the Newcomer hemoglobin attachment. The glass disc was standardized with acid hematin, prepared from hemin crystals as proposed by Elvehjem.[12] The source of light was a Bausch and Lomb Optical Company "Chalet" lamp, equipped with a plate of frosted glass and one of daylight glass. To insure light of constant intensity throughout the experiments, the colorimeter and the lamp were securely fastened on a board. The shortest distance between the filament of the 60-watt Westinghouse bulb and the optical axis of the colorimeter at the mirror was 20 cm.

The Sanford-Sheard instrument was a Central Scientific Company photelometer, model No. 12340. For hemoglobin determinations, the blood was taken up with a 0.1 cc. pipette, the tip of which had been tapered on an emery-wheel. One-tenth cc. of blood was added to 20 cc. of 0.1 per cent Na_2CO_3 solution, which had been measured into 50 cc. flasks from a burette. The gel which formed was broken up by vigorously shaking the flask and was removed by filtration through coarse filter paper. The photelometric readings of these solutions were made within two hours after the solutions had been made.

The new acid hematin photelometric method of hemoglobin determination used was a modification of one described by Elvehjem.[52] It consisted of taking 0.01 cc. of blood and diluting it to the 5 cc. mark of a 1 : 501 blood diluting pipette with 0.4 per cent NH_4OH. The content of the pipette was then transferred into a small test tube, and 0.12 cc. of concentrated hydrochloric acid added, making the actual dilution of blood 1 : 513. The tubes were then corked and slowly inverted at least three times to insure an even distribution of acid and the formation of the insoluble material into larger aggregates. The clear acid hematin solutions were held for at least two hours before photelometric reading was made.

In order to interpret the photelometric readings in terms of hemoglobin, it was necessary first to calibrate the instrument by acid hematin solutions of known concentration. This was done by the following procedure:

Pure hemin crystals were prepared from chicken blood by the method decribed by Elvehjem.[12] The dry crystals were then washed in 95 per cent alcohol, suspended in ether, filtered and dried at 37 C. The crystals were stored in a desiccator over $CaCl_2$. The iron content of the hemin, as shown by ten analyses by the ortho-phenanthroline method[29,49] ranged from 7.60 to 7.87 per cent. The mean value was 7.75 per cent. This value was used in calculating the hemoglobin equivalent of the hematin solutions.

Six sets of acid hematin standards were now prepared, the concentrations of which ranged from 1 mg. to 10 mg. per 500 cc. of solution at intervals of 1 mg. A primary hemin solution was made by dissolving 50 mg. of hemin crystals in a small amount of dilute NH_4OH and adding enough distilled water to bring the volume to 100 cc. The necessary amounts of the primary solution for each

standard were transferred to 500 cc. volumetric flasks, 10 cc. of 5 per cent gelatin solution added, and enough 0.1 N HCl to bring the volume to 500 cc. Two and one-half cc. to 7.5 cc. of concentrated HCl was added to the more concentrated standards to adjust the pH to 1.3.

The Cenco blue No. 1 filter was used in calibrating the photelometer with acid hematin solutions. This filter was found to be more sensitive to small variations in hemin concentration than monochromatic and Wratten filters with transmission bands in the red region corresponding to acid hematin solutions.[33, 39, 44] Photelometer readings of the standard acid hematin solutions were taken and plotted (fig. 1).

The standard acid hematin solutions, as stated above, were prepared by dissolving a known amount of hemin in 500 cc. of fluid. In preparing acid hematin solutions from blood, the same ratio of hemin to volume was maintained by always diluting the blood 500 times. Thus, the amount of hemin in 500 cc. of acid hematin prepared from blood represented the amount of hemin in 1 cc. of blood. Therefore, the amount of hemin per cc. of blood in the acid hematin solution prepared from blood would correspond to the amount known to be present in the standard acid hematin solution which gave the same photelometer reading. In other words, the photelometer having been calibrated with standard acid hematin solutions, the photelometric readings of the acid hematin solution prepared from blood would show the amount of hemin it contained, and from this the amount of hemoglobin per volume of blood could be readily calculated by (1) determining the amount of iron in the hemin and (2) determining the amount of hemoglobin represented by the iron.

As already stated, the iron content of the hemin was found to be 7.75 per cent. The iron content of hemoglobin is well established to be 3.35 per Gm. The actual dilution of blood was 1 part in 513, instead of 1 in 500; therefore, it is necessary to apply a correction factor of $\left\{\frac{5.13}{5.00}\right\}$ 1.026 in these calculations,* which consist in multiplying the mgs. of hemin taken from the calibration curve (fig. 1) by the correction factor of 1.026 to determine the real amount of hemin in 1 cc. of blood. This result multiplied by 0.0775 gives the amount of iron in the hemin. Dividing this by 3.35, the grams of hemoglobin represented by the iron in 1 cc. of blood are obtained. Finally, the result is multiplied by 100 to determine the amount of hemoglobin in 100 cc. of blood. These calculations are expressed in the formula:

$$\frac{(\text{mg. hemin} \times 1.026 \times 0.0775)}{3.35} \times 100 = \text{Gms.}$$

hemoglobin per 100 cc. of blood. Applying this formula to the values taken from the curve of fig. 1, a chart giving the photelometric readings with their corresponding hemoglobin values was prepared for use in routine hemoglobin determinations and is shown in table II.

The alkaline hematin solutions were prepared in the following manner: 0.01 cc. of blood was diluted with 5 cc. of N/10 HCl in a 1 : 501 blood diluting pipette. The contents were then transferred to a test tube and corked. After at least one hour, 0.6 cc. of 5 per cent NaOH was added to convert it to alkaline hematin. This gave a clear solution free from gel or other insoluble material. Photelometric readings were made immediately.

The procedure for calibrating the photelometer with alkaline hematin was similar to the method described for acid hematin, the difference being that NaOH was used in place of HCl in the preparation of the standard hemin solution and in the special filter used.

The primary concentrated hemin solution was made by dissolving exactly 50 mg. of crystaline hemin in a 100 cc. volumetric flask with N/2 NaOH and diluting to the mark with distilled water. The necessary amounts of dissolved hemin were transferred to 500 cc. volumetric flasks with a 10 cc. microburette and diluted to the exact volume with 0.08 N NaOH and distilled water. The pH of the final solution was 11.8.

The filter used for alkaline hematin was made from selected pieces of cellophane, whose transmission corresponded as closely as possible to two of the absorption bands of an aqueous alkaline hematin solution. These were found by Lewin, Miethe and Stenger[33] to be in the red region at 6160A° and in the blue region at 4280A°. The selection of the cellophane was made approximately with a hand spectroscope and three sheets mounted between two plates of glass with nevelite. The relationship between the concentrations of alkaline hematin and the photelometric response is shown in fig. 2.

The formula for calibrating the photelometer for the alkaline hematin method differed only in the correction factor, which was 1.014.

Results of Hemoglobin Determinations of Chicken Blood by Five Methods

1) *Comparison of the variability in the hemoglobin values obtained by each method.*—The procedure for this study was as follows: Ten blood samples were taken in rapid succession from the same bird and tested for hemoglobin content by one of the methods. This procedure was repeated until 100 hemoglobin determinations had been made of 10 birds by each of the Sanford-Sheard, Newcomer (as modified by Schultz and Elvehjem) and Haden-Hauser methods. With the new photelometric acid hematin method, 90 hemoglobin determinations were made of nine birds and 80 determinations of 8 chickens with the new alkaline hematin method.

The Dare method was not included in this comparative study, because each determination must be completed immediately after a blood sample is taken. This would

*This would not have been necessary if the standard acid hematin solutions had been diluted to 513 cc. instead of 500 cc.

make the interval between samples so long that it would be necessary to puncture the veins for each one. Therefore, the studies of this method were limited to single determinations of different individuals in comparison with determinations of the same individuals with the other methods.

To facilitate comparisons of the degree of variability in hemoglobin values given by the various methods, the data were treated statistically and are presented in table III. The variations in hemoglobin values given by the photelometric acid hematin and the modified Newcomer methods were similar and too small to introduce a serious source of error in hemoglobin values, and considerably less than those given by the other three methods. Larger variations were obtained by the Sanford-Sheard method and still larger by the Haden-Hauser and the photelometric alkaline hematin methods. Thus, from the standpoint of consistency in results, the photelometric acid hematin and modified Newcomer methods should be superior.

2) *Comparison of the hemoglobin values obtained by the different methods.*—This study was made with blood taken from 101 Leghorn pullets from 42 to 56 days old. Sufficient blood was obtained from each bird at one time to make duplicate tests for hemoglobin by the photelometric acid hematin, modified Newcomer, Sanford-Sheard, Haden-Hauser, and Dare methods. Hemoglobin determinations with the alkaline hematin method were made upon 29 of the 101 birds in this experiment. The hemoglobin values obtained by all methods of determination were calculated in grams of hemoglobin per 100 cc. of blood. The mean values obtained by each method is given in table IV.

From these data, it is evident that the amounts of hemoglobin indicated by the various methods differed between rather wide limits. The lowest mean hemoglobin value was 7.72 ± 0.91 Gm. obtained with the Dare method. Great difficulty was encountered in matching the shade of red of the blood film with the ruby glass standard —so great, in fact, that it was only by chance that larger variations in the hemoglobin values by this method were not obtained. The same difficulty was experienced by Senty,[53] Olson,[40] Lindsay and associates[34] and Mills,[37] the latter concluding that the accuracy of the Dare method is a little greater than the Tallqvist scale. Mills also reported that the hemoglobin values for human blood given by the Dare instrument were regularly lower (as much as 20.2%) than those obtained by the Van Slyke method. It seems likely that this may also apply to our results with this instrument.

The Haden-Hauser instrument, like the Dare, employs a colored glass standard. It gave only slightly higher hemoglobin values (7.84 ± 0.83 Gm.). This method requires the conversion of the hemoglobin in the blood to acid hematin. The resultant turbidity of acid hematin solution of chicken blood prepared in the manner prescribed for the method made matching of the color of the solution with the glass standard extremely uncertain. The effect of this is clearly shown by the large coefficient of variation obtained from repeated hemoglobin determinations of blood of the same chickens (table III). The clear acid hematin solutions as prepared for the modified Newcomer and photelometric acid hematin methods are not adaptable to the Haden-Hauser method, because the depth of color is insufficient for comparison with the colored glass standard of the instrument. Clear solutions of sufficient concentration cannot be prepared because taking of chicken blood in concentrations of 1 to 10 or 1 to 20 results in the formation of a thick jelly mass from which all of the hemoglobin is not separated and converted into acid hematin upon addition of HCl. The Haden-Hauser instrument, therefore, may be regarded as unsuitable for determinations of hemoglobin in chicken blood, in that accurate readings are difficult and the values indicated by it are too low.

The Sheard-Sanford method gave a somewhat higher hemoglobin value (8.91 ± 0.87 Gm) than the two methods just discussed. In this method, the hemoglobin of the

TABLE III.—Standard Deviation and Variation Coefficients of the Hemoglobin Content of 470 Blood Samples From 47 Birds as Determined by Five Different Methods

	Photelometric Acid Hematin Method		Modified Newcomer Method		Haden-Hauser Method		Photelometric Alkaline Hematin Method		Sheard-Sanford Method	
Series	Sigma (gms.)	V. (%)	Sigma (gms.)	V. (%)	Sigma (gms.)	V. (%)	Sigma (gms.)	V. (%)	Sigma (gms.)	V. (%)
1	0.165	1.45	0.160	2.11	0.323	4.50	0.235	1.47	0.088	1.09
2	0.095	1.25	0.145	1.69	0.415	4.81	0.373	2.50	0.299	2.77
3	0.181	1.95	0.091	1.50	0.431	5.73	0.897	6.65	0.237	2.85
4	0.162	1.66	0.195	2.77	0.225	2.76	0.488	3.92	0.260	3.25
5	0.244	2.31	0.123	1.59	0.329	4.05	0.479	4.01	0.269	3.28
6	...	...	0.104	1.32	0.354	4.48	0.222	1.69	0.164	1.92
7	0.164	1.78	0.159	2.28	0.172	2.24	0.375	2.70	0.138	1.81
8	0.214	2.04	0.193	2.50	0.197	2.43	0.325	2.83	0.246	2.82
9	0.131	1.47	0.148	1.99	0.136	1.78	...	...	0.281	3.27
10	0.228	2.29	0.106	1.38	0.181	2.67	...	...	0.357	4.47
Average	0.176	1.80	0.142	1.91	0.276	3.55	0.428	3.22	0.234	2.75

TABLE IV.—Mean Hemoglobin Content of Blood of 101 Normal Chickens, 42 to 56 Days of Age, Obtained From Determination in Duplicate by Each of the Six Methods

	Methods					
	Dare	Haden-Hauser	Modified* Newcomer	Sheard-Sanford	Photelometric Acid Hematin	Photelometric Alkaline Hematin**
Mean Gm. of hemoglobin per 100 ml of blood	7.72	7.84	9.71	8.91	9.83	12.13
Standard deviation	0.91	.83	0.93	0.87	1.00	0.99

*Actual reading × correction factor for disc of 1.33.
**Mean hemoglobin value of 29 chickens.

blood is converted into oxyhemoglobin by laking the blood and then determining the amount of hemoglobin with a photelometer, which has been calibrated previously against hemoglobin determinations of mammalian blood by the Van Slyke method. Since the Van Slyke procedure is not applicable to chicken blood, the calibration chart for mammalian blood must be used in applying the Sheard-Sanford method to chicken blood. The gel in laked chicken blood probably traps some of the hemoglobin, and the reading obtained, therefore, does not represent all of the pigment present in the blood. Another disadvantage of the Sheard-Sanford method for chicken blood is the variability in amounts of hemoglobin indicated, as was shown by the repeated determinations of blood of the same chicken (table III). A similar variability was reported by Olson.[40]

The modified Newcomer and photelometric acid hematin methods yielded hemoglobin values nearly alike, *i.e.*, 9.71 ± 0.93 and 9.83 ± 1.00 Gm. per 100 cc. of blood. These two procedures also gave the most consistent results in the series of repeated determinations of the same blood (table III). Since the standards for both methods are acid hematin solutions of known concentration, they should accurately portray the hemoglobin content of acid hematin solutions prepared from the blood of chickens. The essential difference between the two

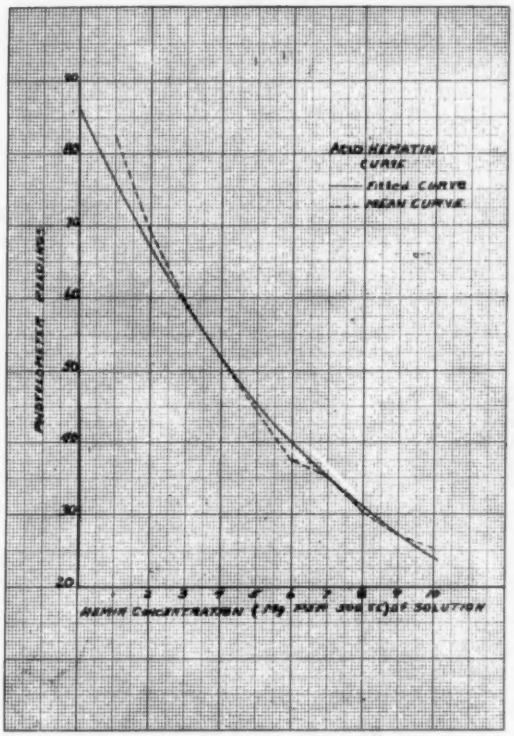

CHART I—Photelometer readings of acid hematin solutions of known hemin content.

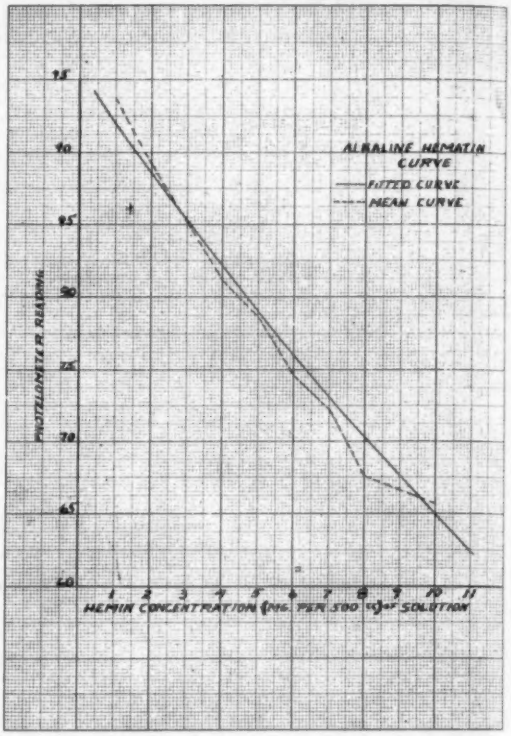

CHART II—Photelometer readings of alkaline hematin solutions of known hemin content.

methods is that readings of the former are made visually with a Duboscq colorimeter equipped with a Newcomer disc and those of the latter with the photelometric colorimeter. In choosing between the two, preference might be given to the latter, because it is not subject to error resulting from variation in the ability of different individuals to match colors or to differences in type or intensity of the light used with the Newcomer instrument.

The hemoglobin values obtained by the photelometric alkaline hematin method (12.13 ± 0.99 Gm. per 100 cc. of blood) averaged 2.25 Gm. more and showed greater variation than was obtained with any other method (table III). The fact that greater variations in hemoglobin values were given by this method than with others suggests that the higher average values are due to the presence in the solution of non hemoglobin substances in fluctuating amounts. The full elucidation of this point is dependent upon the determination of the nature of the substances removed in preparation of acid hematin solutions of blood, since the presence or absence of this material is the only essential difference between the alkaline and acid hematin preparations.

SUMMARY

1) Comparative studies were made of the adaptability and the relative accuracy of the Dare, Haden-Hauser, and Sheard-Sanford methods, the modified Newcomer method, a new photelometric acid hematin and photelometric alkaline hematin method for the determination of hemoglobin in chicken blood. The relative consistency of performance of each method was determined by repeated tests of blood of the same individuals. The hemoglobin values given by each method were obtained by duplicate tests of 101 White Leghorn chicks, 6 to 8 weeks old.

2) The modified Newcomer method and the new photelometric acid hematin method gave the most consistent results

and are considered as most suitable for the determination of hemoglobin in chicken blood.

3) The hemoglobin content of the normal 6- to 8-week-old White Leghorn chickens was established as 9.71 ± 0.83 Gm. and 9.83 ± 1.00 Gm., respectively, by the modified Newcomer and photelometric acid hematin methods.

4) The Dare, Haden-Hauser, and Sheard-Sanford methods were found to be unsatisfactory for determination of hemoglobin in chicken blood.

5) The photelometric alkaline hematin method gave variable hemoglobin values, which averaged 2.25 Gm. per 100 cc. of blood higher than those obtained with the modified Newcomer and photelometric acid hematin methods. Both the variability and higher hemoglobin values are believed to be due to the presence of nonhemoglobin constituents of blood in the alkaline hematin solution.

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Recovery of Clostridium Sordellii From Cattle and Sheep

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DURING THE PAST 20 years, *Clostridium sordellii* has been recovered six times incident to the bacteriological examination of tissues from cattle and sheep at the University of Nevada.

This extremely pathogenic sporulating anaërobe was first described by Sordelli, of South America, in 1922.[1] He obtained it from two out of 11 fatal cases of gas gangrene in man. Sordelli named it *"Bacillus oedematiens sporogenes."*[2] In 1927, Meleney, Humphreys, and Carp reported the recovery of a similar pathogen from a fatal operative wound infection in a woman, and also from surgical catgut.[3] Being unaware of Sordelli's work, they named their organism *"Clostridium oedematoides."* Hall and Scott,[4] Hall,[5] and Hall, Rymers, and Jungherr[6] examined the cultures of Sordelli and Meleney from human sources and two novyi-like cultures isolated from cattle in Nevada and declared they were culturally and serologically identical. Hall and Scott then suggested the binomial name *"Bacillus sordellii"* for this new species. Subsequently, Hall and Gray[7] reported a fatal case of septic peritonitis with terminal bacteremia in a man due to *Cl. sordellii* infection.

We recovered *Cl. sordellii* the first time in 1921 from the liver infarct of a bull dead of bacillary hemoglobinuria (red water disease) near Reno. The second culture was recovered under similar circumstances in 1923 from the liver infarct taken from a cow on a ranch about 50 miles from the source of the first culture. It was soon established that the two cultures of this anaërobic organism were identical in respect to morphological and cultural features and pathogenicity, but were immunologically distinct from other then recognized species of sporulating pathogenic anaërobes. They were found to be extremely pathogenic for all laboratory animals, as well as for horses and cattle. After considerable work, they were regarded as accidental invaders having no relation to the etiology of bacillary hemoglobinuria under investigation at that time.[8]

Being aware of the extremely pathogenic nature of this sporulating anaërobe for all laboratory and some farm animals, from our own experience, as well as for man, as reported by Meleney and Hall, we anticipated that it might sometime be encountered as a primary pathogen among farm animals. To date, we have recovered six cultures of *Cl. sordellii* from animal sources in western Nevada. Five of them came from cattle and one from sheep. Three of the cattle strains were isolated along with either *Clostridium hemolyticum* or *Clostridium welchii* from the liver infarcts or other tissues of cattle dead of bacillary hemoglobinuria. Another cattle strain was

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recovered along with *Clostridium chauvei* from the heart blood of a calf dead of atypical blackleg. The lesions found in calves in this outbreak consisted of a severe hemorrhagic gastroenteritis with blackleg-like lesions in the pillars of the diaphragm. Recently, another strain of *Cl. sordellii* was recovered from a fatal post-vaccination infection among Hereford calves, which will be described in another paragraph. One nonpathogenic strain was recovered along with *Clostridium septicum* from a sheep dead of a braxy-like disease near Reno. Although nonpathogenic, the sheep strain is culturally and serologically related to the cattle strains, reciprocal agglutination occurring between them. Hall also encountered a nonpathogenic strain among cultures submitted by Sordelli.

In March, 1941, we encountered what appears to have been a fatal infection due to *Cl. sordellii* among Hereford calves, following vaccination with a sterile formalinized blackleg vaccine from a reputable manufacturer. No reflection can be cast on the vaccine itself. Three calves died about 48 hours after vaccination. Two unmutilated carcasses were examined about six hours after death. No characteristic lesions of blackleg were found, although both carcasses were moderately bloated, and bloody discharges occurred from natural body openings. Other than slightly gaseous, putty-colored livers, the visceral lesions were not significant. The principal lesion in both calves consisted of a moderately emphysematous, mildly hemorrhagic edema involving mainly the intermuscular connective tissue along the left side of the neck and dorsal region. The neighboring lymph glands were extremely edematous and hemorrhagic. Portions of the shoulder and neck lesions were packed in borax and brought to the laboratory. Guinea pigs inoculated with lesion exudate diluted with equal part of physiological sodium chloride solution were dead in 16 hours with an extensive hemorrhagic edema involving the entire abdominal and thoracic region, accompanied by a hemorrhagic gastroenteritis. *Cl. sordellii* was recovered in pure culture from the heart blood of the inoculated guinea pigs. *Cl. sordellii* and *Clostridium sporogenes* were isolated and separated in cultures made directly from the original lesion fluid of the calves. Careful search failed to disclose the presence of any other anaërobic pathogens, such as *Cl. chauvei*, *Cl. septicum*, or *Cl. welchii*.

As *Cl. sordellii* had been recovered on two occasions about 18 years ago from cattle tissues originating on a neighboring ranch, it is believed to be native to the soils of that and other regions in western Nevada. As skin disinfection is not customarily practiced during routine vaccination of cattle in our region, it is presumed that *Cl. sordellii* was present on the hair or skin of some of the cattle and was mechanically introduced into the tissues at the moment of vaccine injection on the left side of the neck.

Cl. sordellii possesses many morphological and cultural features common also to *Clostridium novyi*, *Cl. sporogenes*, and to a lesser extent, *Cl. hemolyticum*. Although the microbiological features were quite fully described by Hall and others more than 12 years ago, it appears appropriate to repeat some of the salient features, as well as to report additional ones not heretofore given. Our observations have been based entirely on strains of *Cl. sordellii* recovered directly from animal sources, but no appreciable differences were noted between them and those from human sources isolated by Sordelli; Meleney, Humphreys, and Carp; and Hall.

Cl. sordellii is a nonmotile, sporulating anaërobic rod, 1 x 3.5-4.0 microns. Young cultures 12 to 18 hours old are strongly gram-positive. Spores are usually located in a central or subterminal position. Although it is an obligate anaërobe, it grows rapidly and luxuriantly on all mediums suitable for anaërobes, such as the cooked-meat mediums or the sodium thioglycollate-agar mediums recommended by Brewer.[9] A very characteristic valeric odor emanates from cultures in 20 to 48 hours, which in itself

[9]Brewer, J. H.: A Clear Liquid Medium for the Aërobic Cultivation of Anaërobes. J. Bact., xxxix (1940), p. 10.

is sufficient to indicate the presence of *Cl. sordellii* even among a mixture of other anaërobes.

The pleomorphism of deep agar colonies may be quite confusing. They may appear as biconvex discs, multiplanate discs, disc types with eccentric fluffs, or dense feathery types. Extensive fracture of the agar column may occur with only 10 or 12 colonies in a deep agar culture tube. Its proteolytic activity in cultures is pronounced and more varied than other pathogenic sporulating anaërobes.

The meat particles in cooked-meat mediums first become bright red, later darkened in color, and are partially digested or gelatinized. White crystals, resembling tyrosin in respect to microscopic crystalline structure and alcohol solubility,[10] appear in old meat cultures after 10 days or longer, developing best at room temperature. Black pigment begins to appear around the meat particles in cooked-meat mediums about the third day. A similar veil-like stratum of black pigment also appears in other fluid mediums, especially those containing sodium thioglycollate and agar, in the absence of cooked meat.

The following tabulation sets forth the main cultural and biochemic features. Where necessary, anaërobic conditions were obtained by vaspar seals or the alkali pyrogallate method.

Gelatin was liquified in 48 hours, but not blackened.

Purple milk was clotted in 24 hours, followed by separation of whey and gradual but incomplete digestion of the clot in 5 to 7 days.

Coagulated serum showed pitting of the surface, followed by gradual liquefaction and blackening of residual clot in 5 to 7 days.

Indol was strongly positive after 18 to 24 hours incubation in liver digest cultures or those containing proteose peptone after the Erlich-Bohme method.[11]

Hydrogen sulfide was produced in large amounts, beginning at the fourth hour of incubation, as indicated by sterile strips of lead acetate test paper inserted along the cotton plugs.[12]

Methyl red was positive, but the Voges-Proskauer reaction was negative, and nitrates were not reduced.

Hemolysis of red cells was rapid and complete in 4 to 6 hours after inoculation into deep agar dilution cultures containing 1 per cent washed sheep red cells.

Carbohydrates fermented in 24 hours were dextrose, levulose, and maltose.

Carbohydrates not fermented at the end of 7 days were galactose, lactose, saccharose, mannite, arabinose, xylose, inulin, salicin, dulcitol, and glycerol.

Usually, all substances fermented by the various pathogenic sporulating anaërobes are reduced during the first 24 hours of incubation, provided the basal medium is adequate and proper anaërobic conditions prevail.

A very potent, moderately stable, filtrable toxin is produced in fluid mediums reaching a maximum concentration in 24 to 36 hours. It is inactivated at 70 C. in 20 minutes and rendered anatoxic by 0.25 per cent formalin in 2 days at 37.5 C. Formalinized cultures are antigenic.

As *Cl. sordellii* closely resembles *Cl. novyi* and *Cl. sporogenes* in many respects and *Cl. hemolyticum* to some extent, its presence might easily escape detection in a casual examination of tissue exudates or a mixed culture of anaërobes. Except in the post-vaccination infection, it occurred in a minority to other anaërobes, judging from the proportion of colonies in deep, agar-shake cultures made from the original material. Its ability to induce a rapidly fatal toxemia may compensate for the numerical minority.

The anatomical lesions occurring in laboratory animals injected with toxic *Cl. sordellii* cultures do not present any special features which would distinguish them

[10]Hawk, P. B., and Bergeim, O.: Practical Physiological Chemistry, 11th ed., P. Blakiston's Son and Company, Inc., Philadelphia (1937), pp. 120-122.

[11]Pure Cult. Study of Bact., x (1942), pp. 11-12.

[12]ZoBell, C. A., and Feltham, C. B.: A comparison of Lead, Bismuth and Iron as Detectors of Hydrogen Sulphide Produced by Bacteria. J. Bact., xxvii (1934), pp. 169-178

from lesions induced by some of the other gas edema-producing anaërobes, especially *Cl. novyi*. Connective tissue is primarily affected with a spreading gelatinous edema. The coloration of the edema is roughly proportional to the dose injected. Small doses, 0.05 to 0.1 cc., even though invariably fatal for guinea pigs, usually cause thick, clear, gelatinous local lesions resembling those produced by some strains of *Cl. novyi*. This lesion also resembles the local reaction of guinea pigs or rabbits following inoculation with some freshly isolated field strains of *Bacillus anthracis*.

Larger doses, 0.2 to 0.5 cc., when injected into the subcutis of the abdomen of guinea pigs, usually cause death in 16 or 18 hours, accompanied by a diffuse, intensely hemorrhagic edema of the entire abdominal wall, reddening of the contiguous musculature, and a variable degree of hemorrhagic enteritis. *Cl. sordellii* becomes bacteremic in all laboratory animals and may occasionally become bacteremic in cattle along with *Cl. chauvei*, as we have cited. Horses are particularly susceptible to *Cl. sordellii* infection. Small doses, 0.5 cc., of a toxic culture may result in a massive local reaction, high fever, 105 F., severe toxemia, and death. Even germ-free Berkefeld culture filtrates will cause severe local reactions in horses.

It is almost impossible to immunize rabbits with living organisms, even though they are rendered potentially free of toxin by repeated washing. The rabbits usually die in a few days with a bacteremia. However, we were successful in producing agglutinating serums in rabbits with four strains of *Cl. sordellii* by starting with 1 cc. of formalinized whole cultures injected subcutaneously, followed by formalinized cultures given intravenously at weekly intervals. A very potent antitoxic serum can be produced in horses and is now included in some polyvalent anaërobic serums on the market.

Cross-agglutination occurs among all of the strains we have recovered to date. Antitoxic serum produced in horses in 1922, using the first strain recovered, protects against all other pathogenic strains from animal sources as well as the original Sordelli strains from South America, thus indicating a monovalent group in respect to antigenic structure.

As far as we are aware, in every instance where *Cl. sordellii* has been recovered, it was associated with a fatal effect on the subject, and the identity or presence of this organism was not established nor suspected prior to death. It has been recovered in widely separated localities in the western hemisphere.

From the reports of Sordelli, Meleney, and our own observations, it is obvious that a very small number of *Cl. sordellii* organisms, accidentally implanted in surgical or other wounds, may result in a rapidly fatal infection. It may also invade tissues in the absence of surgery or mechanical traumatism, as reported by Hall and Gray, or concurrently with other pathogenic anaërobes, as occurred in several instances in cattle and sheep which we have described. Sporulating forms will survive heating at 90 C or freezing at —70 C., as well as dehydration for many years. Meat medium cultures which had entirely dried out after 10 to 12 years storage were readily revived by adding sterile liver digest broth and were found to be fully virulent. It may be expected, therefore, to survive almost indefinitely in soils or other organic material. This extremely pathogenic anaërobe occasionally may play a rôle heretofore unsuspected in obscure anaërobic infections of animals as well as man.

By utilizing some of the knowledge now available, such as its rapid growth in mediums suitable for anaërobes, the agglutination test with young vegetative cultures, and serum protection tests with laboratory animals, it might be feasible to make a diagnosis early enough with cultural material taken from an external traumatic wound to institute specific biologic therapy with the hope of saving the life of the victim. Compared to the other well-known pathogenic species of the genus Clostridium, we would consider *Cl. sordellii* to be one of the most dangerous, and perfectly

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Field Test of Various Anthelmintics Used for the Treatment of Haemonchosis

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EVER SINCE the discovery by Hutcheon[1] that copper sulfate would remove stomach worms from sheep, much has been written on the subject. Unfortunately, there is little agreement among the writers as to the posology, effective adjuvants, or method of administration of the drugs. This is due to a variety of causes, some of which will be discussed in this paper. As a result of this disagreement, the veterinarian and the stockman are confronted with many recommendations and formulas. Since these compounds varied widely in composition and price, it was decided to test them to see if any were more efficient than simple copper sulfate against *Haemonchus contortus*. These tests were carried out on heavily infected farm flocks in north central Kansas during the spring, summer and fall of 1941. Apparently, this area presents ideal conditions for the development of pure *H. contortus* infections. Other "nemic" parasitisms causing disease in sheep are rare. Many flocks in this territory are purchased from regions where other helminthiases are prevalent, but the ecological relationships apparently combine to eliminate all but the "twisted wire worm" as a potent source of danger. The tendency of farmers in the area to use the sheep primarily as a clean-up flock, or to keep them on small pastures which become inadequate during the latter part of the summer and rarely to supplement these deficient diets, allows haemonchosis to become prevalent and to appear in its purest clinical form. The disease is commonest from mid-August until frost, although sporadic outbreaks occur at other times of the year.

Contribution No. 93, Department of Veterinary Medicine, Kansas State College.

[1]Hutcheon, D.: Bluestone for Wireworms, Agr. Jour. Cape Colony, iv (1892), p. 240.

Materials and Methods

Contact was made with various flock owners through the coöperation of their local veterinarians. Fecal samples from 10 sheep in each flock were obtained directly from the rectum and the sheep tagged for future identification. It was found easier to obtain the samples if the sheep were confined in a small area and allowed to feed overnight. These samples were examined by a modified Stoll-McMasters technique (Whitlock).[2] If this preliminary examination revealed that the flock was heavily infected, it was treated with two or more anthelmintics at the same time. One of these treatments was always a simple copper sulfate solution of either 1.7 or 10 per cent strength. The other treatment was the compound which was to be tested against copper sulfate. Just before the animal was treated, a fecal sample was taken from the rectum and the animal marked for identification. The fecal samples were taken to the laboratory and frozen until they could be counted. From one to three weeks after treatment, the flock was revisited and fecal samples of those shown to be heavily infected on the first visit were obtained and examined by the dilution egg-count technique. Although all the sheep in a given flock were treated, no data were kept on any animals showing an initial egg count below 2,000 e.p.g. Two reasons for this were:

1) As Peters and Lieper[3] have shown, dilution egg counts are distributed in the Poisson fashion. This means that the standard error of such counts is very large when dealing with small numbers.

2) Taylor[4] has indicated that 2,000 to 6,000 e.p.g. represents the critical egg-count level in stomach-worm disease.

[2]Whitlock, J. H.: Studies Upon Strongylus Vulgaris. V In vitro Toxicity of Copper Sulphate and Cupric Halides, Jour. Parasit. xxiv (1940b), pp. 49-57.

[3]Peters, B. G., and Leiper, R. T. L.: Variation in Dilution Counts of Helminth Eggs, Jour. Helmin., viii (1940), pp. 117-148.

[4]Taylor, E. L.: Diagnosis of Helminthiasis by Means of egg Counts with Special Reference to Red-worm Disease of Horses, ii (1939), pp. 895-898.

Clostridium Sordellii

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capable of doing all the damage characteristic of other pathogenic members of this genus.

TABLE I (Part I).

FLOCK	DATE OF TREATMENT	DRUG	SATISFACTORY	UNSATISFACTORY	TOTALS OF COPPER SALTS FOR EACH FLOCK: SATISFACTORY	TOTALS OF COPPER SALTS FOR EACH FLOCK: UNSATISFACTORY	
A	5/6	Cunic* Sol.	9	5	..	..	...
		Cuform Sol.	7	4	..	..	...
		Cu Sol. 1.7%)	11	3	..	..	...
A	5/20	Cu Sol. (1.7%)	7	16	..	..	...
		Cunic Cap. *A*	7	15	41	43	8.899
B	8/20	Cu Sol. (1.7%)	7	2	..	..	...
		Cu-Ars Sol.	7	1	..	..	...
B	9/12	Cu Sol. (10%)	1	1	..	..	...
		Cu-Ars Sol.	1	3	..	..	...
B	9/27	Cu Sol. (10%)	4	1	..	..	...
		Cu-Ars Sol.	2	2	22	10	.264
C	9/19	Cu Sol. (1.7%)	5	1	..	..	...
		Cunic Caps. *B*	4	3	..	..	...
C	9/23	Cu Sol. (10%)	1	2	..	..	...
		Cunic Caps. *B*	1	3	11	9	.770
D	11/7	Cu Sol. (10%)	15	5	..	..	...
		Cu-Ars Sol.	17	3	..	..	...
		Cunic Caps. *B*	10	6	..	..	...
		Cunic Caps. *C*	8	2	..	..	...
D	11/11	Pheno-Bol.	24	0	..	..	...
		Cu Sol. (10%)	20	3	..	..	...
D	11/13	Cu Sol. (10%)	22	5	..	..	...
		Cunic Caps. *C*	15	6	..	..	...
		Pheno-Bol.	18	0	..	..	...
		Pheno. Sol.	25	0	87	27	7.118
				Totals	161	89	17.069**

*Cunic = Coined term for copper-nicotine mixture.

In the course of these experiments, we were able to confirm the findings of both investigators.

Several different methods of recording the efficacy of the various drugs were tried. Because the inaccuracies inherent in the dilution egg count prevent refined statistical analysis, it was felt that division of the treated animals into satisfactorily and unsatisfactorily treated groups upon some simple basis would provide the most practical method of presenting and analyzing the data. Since Taylor (*op. cit.*) has suggested 2,000 e.p.g. as the danger line between pathogenic and nonpathogenic infections, a reduction below that level should indicate a satisfactory treatment. Gordon[9] has suggested that a 70 per cent reduction in e.p.g. count represented a satisfactory treatment. Both criteria were tried and agreed remarkably well with one another in their estimate of drug efficacy, although some discrepancies appeared.

[9]Gordon, H. McL.: The Efficiency of Copper Sulphate and Carbon Tetrachloride Against Haemonchus Contortus in Adult Sheep, Aust. Vet. Jour., xv (1939), pp. 118-120.

The present paper uses the 2,000 e.p.g. level as the mark between a satisfactory and unsatisfactory treatment for the following reasons:

1) The error is smaller, since it is kept to those infections which, before or after treatment, are at or near the 2,000 e.p.g. level.

2) It takes full notice of those cases of a heavy infection with a satisfactory reduction in egg count on a percentage basis, but unsatisfactory on the basis of the actual worm burden.

3) The use of the percentage interpolates an extra calculation between the experimental data and their analyses, with no appreciable benefit.

Since the sheep for the different groups were selected at random, as was to be expected, the size of the initial infections was well balanced between each group.

The copper sulfate and formate used in these tests were chemically pure and the tobacco dip (containing 40% nicotine sulfate) used to make the cunic solution in

TABLE I (Part 2).

NAME OF DRUG	SOURCE	COMPOSITION	DOSE	PRICE PER 100 ADULT SHEEP
Cunic Sol.	Compounded by us	1.7% $CuSO_4$ (C.P.) 1.7% tobacco dip (40% nicotine)	Lambs 1-3 mo. 2-5 cc. Lambs 3-6 mo. 30 cc. Lambs 6-12 mo. 45 cc. Adults 60 cc.	$00.475 Approx.
Cu Sol.	Compounded by us	1.7% $CuSO_4$	Same as Above	$00.12
Cu Sol.	Compounded by us	10% $CuSO_4$	1 cc. per 10 lb. of body weight	$00.12
Cuform Sol.	Compounded by us	1.535 Cu formate	Same as cunic solution	
Cunic Cap. *A*	Veterinary supply firm *A*	Ext. Kamala 8.33 gr. Copper sulfate 10.00 gr. Nicotine sulfate 2.00 gr. In shiny red capsules	1 capsule for lamb 2 for adult	$4.20
Cunic Cap. *B*	Veterinary supply firm *B*	Ext. Kamala 20 gr. Nicotine sulfate 6 gr. $CuSO_4$ 20 gr. In cylindrical tablets	½ capsule for lamb 1 capsule for adults	$2.60
Cunic Cap. *C*	Veterinary supply firm *C* and livestock remedy house *A* put out apparently same capsule	Nicotine sulfate 1 gr. $CuSO_4$ 17.5 gr.	1 capsule for ewe	$1.00
Cu-Ars. Sol.	Veterinary supply house *A*	Arsenic trioxide 1.00% copper sulfate with Formalin 7% in special non-irritating base	1 cc. per 10 lb. of body weight	Approx. $1.00
Pheno Sol.	Veterinary supply house	Phenothiazine 12 gr. per wetting agent and water Q.S. 1 oz.	1 oz. under 50 lb. 2 oz. over 50 lb.	$8.60*
Pheno Bol.	Veterinary supply house	Large boluses containing 12 g. phenothiazine	1 bolus to lamb 2 boluses to adult sheep	$16.00*

*These and other experiments show 1/2 recommended dose of phenothiazine often effective in ovine haemonchiasis.

test 1 was obtained from a reliable source. The composition, price, dose and type of source of the various formulas tested are listed in table I. In every case, the directions of the manufacturer were carefully followed in administering the drug with a single exception. Each maker had detailed directions about access of the sheep to feed and water before and after treatment. These were ignored for the following reasons:

1) The experimental evidence upon which such recommendations are based is extremely shaky.

2) It is not considered sound therapeutics to take food from a sick, weakened animal suffering from a dietary deficiency.

3) Swales[5] has shown that starvation for the periods usually recommended has no effective influence upon the abomasal contents of sheep.

4) Since phenothiazine can be given to an animal on full feed without loss of effect, the present compounds should be tested under like conditions.

[5]Swales, W. E.: Preparation of Sheep for Anthelmintic Medication, Can. Jour. Comp. Med. and Vet. Sci., ii (1938), pp. 57-58.

Preliminary to the cunic, copper formate and copper sulfate solutions used in these tests, a mouth spray of 1 to 2.5 cc. of 10 per cent copper sulfate was administered to stimulate contraction of the oesophageal groove, as suggested by Monnig.[6]

The flocks used in these experiments were typical farm or feeder flocks consisting mostly of "westerns." Flock *A* at the time of treatment showed only the ewes to be heavily infected with *H. contortus*. Flock *B* had both ewes and lambs heavily infected, and flock *C* had only lambs showing a heavy infection. Flock *D* was a feeder-lamb flock from Texas. It was impossible to obtain accurate mortality figures from any of these flocks, but with the exception of flock *C*, animals were available for postmortem examination, and a diagnosis of haemonchosis could be made. Some egg-count data were lost because of lack of fecal samples or time, but these should not influence the results since the losses were due to chance.

ANALYSIS OF EXPERIMENTAL DATA

Table I, which summarizes the results of these experiments, shows no evidence that the addition of formalin and arsenic, nicotine sulfate, or formate to soluble copper salts increases the efficacy of the copper against *H. contortus*. In the case of the cunic preparations there is no evidence that either the capsule or the drench was more effective than the simple copper sulfate solution used at the same time. Even when all the data from the several tests of nicotine or arsenic are combined and compared with the proper simple copper sulfate solution by means of chi-square, no evidence of increased efficacy can be demonstrated.

These data confirm the suggestion of Hardy and Schmidt[7] that nicotine does not supplement copper effectively. It is, of course, possible that the administering of the drugs without starvation depressed the efficacy of the fortified compound and not the simple copper sulfate solutions, but that is unlikely. What is more probable is the existence of some factor, possibly the erratic reaction of the oesophageal groove, which prevents all or part of the drug in certain animals from reaching the parasites.

Table I also reveals that phenothiazine is significantly more effective than copper compounds. This confirms the reports of numerous other investigators.

DISCUSSION

Before it is possible to discuss the therapy of a disease, it is necessary to identify its various manifestations. Following the example provided by Cort[8] in ancylostomiasis, we prefer to distinguish between acute and chronic haemonchosis. In any heavily infected flock, both types of the disease occur as well as infections which apparently cause no disturbance whatsoever. For the purposes of subsequent discussions, we define acute haemonchosis as an acute disease caused by the activities of *H. contortus* in the abomasum, and characterized by a marked anemia, pronounced symptoms of subcutaneous edema, lassitude, loss of appetite, and constipation. The acute form of the disease in Kansas is often complicated by respiratory infections, screwworm infestations, and sometimes tapeworm infections. It occurs in young animals and mature animals on a deficient diet. The chronic form of the disease is often difficult to ascertain clinically. This, the commonest form, may flare up into the acute, particularly if anything occurs to overcome the host's resistance to the parasite. The chronic type grades insensibly into the mere infection. Since it is difficult to say exactly how many worms will make a sheep sick, we prefer to simplify the problem and assume that any infection producing 2,000 e.p.g. is causing sufficient blood loss to be a detriment to the host. A chronic infection, therefore, is any infection producing over 2,000 e.p.g. without causing marked symptoms characteristic of the acute form.

On the basis of the present experiments,

[6]Monnig, H. O.: On the Toxicity of Nicotine for Sheep and the use of Nicotine Bluestone Drench for Worms in Ruminants, Res. de Med. Trop. y Parasit. Bact. Clin. y Lab., iii (1935), pp. 3-10.

[7]Hardy, W. T., and Schmidt, H.: Stomach Worms in Sheep and Goats, Tex. Agr. Exp. Sta. Bull. 448 (1932).

[8]Cort, W. W.: Variations in Hookworm Disease, Jour. Parasit., xix (1932), pp. 142-147.

copper sulfate should be expected to remove satisfactory numbers of *H. contortus* in the majority of cases. Because of the low cost, toxicity and ease of administration, 10 per cent copper sulfate solution in the dosage of 1 cc. per 10 lb. of body weight preceded by 1 to 2.5 cc. of the same solution, depending upon weight, is the treatment of choice whenever the problem consists solely of removing *H. contortus*. This means that it is recommended in chronic haemonchosis and as the drug to be used in monthly treatments to prevent the disease. There is no evidence of fortification; hence, no justification for adding nicotine, formate, formalin or arsenic to copper in the treatment of haemonchosis. Although Gordon[9] has shown that nicotine and arsenic fortify the action of copper against immature forms of *H. contortus*, an animal which really needs to have the immature forms of the parasite destroyed is probably too weak to tolerate copper, nicotine, or arsenic.

Likewise, on the basis of the present experiments, copper sulfate will fail to remove *H. contortus* in satisfactory numbers from an appreciable number of infected animals. Therefore, whenever the problem is fundamentally that of saving the life of an animal suffering from haemonchosis, copper sulfate is decidedly not the drug to use. Particularly is this true in view of the fact that acutely ill animals will sometimes die even though the worms have been removed by copper therapy. Phenothiazine will likewise sometimes fail in acute cases of stomach worm disease, but as far as the present evidence goes, it does not fail as often as copper sulfate. Therefore, it is guardedly recommended for treatment of acute haemonchosis. Since phenothiazine is expensive and difficult to administer, it would be good policy for the practitioner to use it only in the acute cases within an infected flock.

Theoretically, rational treatment of acute haemonchosis would include restoration of the lost blood and improvement of the diet to correct its deficiencies.

The conclusions reached in the foregoing discussion are not in agreement with those of many other investigators. The chief reason for the disagreement is the fact that few workers with anthelmintics take any account of secular variation. Secular variation is the variation in the effectiveness of a drug when it is tested on different sample populations. Thus, in the first visit to flock *A* only 3 out of 14 of the treatments were unsatisfactory, while on the second treatment with the same drug under apparently identical conditions 15 out of 23 were unsatisfactory. When tested by chi-square this difference is significant. The same significant difference shows up when the total effectiveness of all the drugs used in the two treatments is compared. Each of the four flocks differed significantly in their reaction as a whole to the copper anthelmintics, the chi-square analysis in table I demonstrating this point.

TABLE I (Part 3).

	SATISFACTORY	UNSATISFACTORY	
Cunic Compounds	54	40	
Simple $CuSO_4$ Sols.	61	32	
		= 1.309	.. $df = 1$
Cu-Ars Sol.	27	9	
Simple $CuSO_4$	27	9	
		= non-significant	
Phenothiazine	67	0	
Copper Anthelmintics	57	14	
			= 16.12**

This analysis of the present experimental data conclusively demonstrates the existence of secular variation in anthelmintic tests. Its existence, therefore, implies that no anthelmintic tests to compare drug or dose efficacy are valid unless they are done at the same time on approximately the same population of animals. There is a possibility that certain anthelmintics under ideal laboratory conditions evidence no secular variation (Whitlock and Bliss 1942), but such cases in pharmacological literature of any sort are rare. The cause of this secular variation is obscure. It might be due to the sheep carrying varying numbers of immature *H. contortus* which Gordon[10] has shown to be refractory to copper sulfate

[10]Gordon, H. McL.: Anthelmintic Efficiency against Immature Haemonchus Contortus, Aust. Vet. Jour., xv (1939), pp. 57-66.

therapy. If these become mature following treatment, an erroneous conception of the efficacy of the drug might be obtained. However, nicotine sulfate is supposed to fortify the action of copper against immature forms, according to the same writer. The lack of evidence of such fortification would seem to indicate that the secular variation was not primarily due to immature forms of the parasite. The secular variation might be due to the same obscure factors responsible for its appearance with copper nematocides *in vitro* (Whitlock[11]), but this is dubious since these compounds *in vitro* can be fortified. The most probable cause of the secular variation is the fact that the efficiency of the oesophageal groove reflex undoubtedly varies from time to time. In support of this theory is the fact that the most pronounced secular variation occurred in flock *A*, which consisted of aged ewes. As Monnig and Quin[12] have shown, such animals have a poor oesophageal groove reflex.

[11]Whitlock, J. H.: A Practical Dilution Egg-Count Procedure, J.A.V.M.A., xcviii (1940a), pp. 466-469.

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[12]Monnig, H. O., and Quin, J. I.: Studies on the Alimentary Tract of the Merino Sheep in South Africa, II Investigations on the Physiology of Deglutition, Onderstepoort Jour. Vet. Sci., v (1935), pp. 485-489.

The Pathogenicity of Streptococcus Agalactiae for Mice and Treatment with Sulfanilamide and a Derivative

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THE NAME *Streptococcus mastitidis* designated a group of apparently similar organisms isolated in cases of bovine mastitis. With the aid of improved serological and biochemical methods, various investigators have identified the different organisms and types within this group. In 1932, Minett, Stableforth and Edwards,[1] who contributed largely to this clarification, and several German investigators designated as *Streptococcus agalactiae* that member of the group which they considered the most frequent etiologic organism found in chronic bovine mastitis.

The organism used in our experiments was recovered from a recent case of bovine mastitis and was held in culture for two months. It conforms to Group 1 *(Str. agalactiae)* of Minett,[2] and Group B by Lancefield's precipitation test. One cc. of the bouillon culture supplied to us, inoculated intraperitoneally, was nonpathogenic for white mice. This was in agreement with the majority of investigators,[3] who have claimed *Str. agalactiae* to be of low pathogenicity for mice.

Pathogenicity.—The bouillon culture was subcultured in neopeptone broth. Using an adaptation of Long's[4] method, passage of the organism through mice was done in the following manner:

One cc. of the neopeptone broth subculture was injected intraperitoneally. If the animal did not die within 36 hours it was sacrificed and its heart was cultured in neopeptone blood broth. This was incubated for 14 hours at 37 C. and 1 cc. of the culture was inoculated into a second mouse. This procedure was repeated four times. Upon inoculating the fifth mouse, if death did not occur within 7 hours, the animal was killed and its heart cultured in neopeptone blood broth and incubated for 14 hours at 37 C.

Two separate pathogenicity tests were made on 40 mice. Each mouse was inoculated intraperitoneally with 1 cc. of neopeptone blood broth culture. All of the 40 mice died within 24 hours and the organism was recovered, in the majority of instances in pure culture, from all of ten hearts selected at random.

In order to eliminate the possibility of the medium acting as the lethal agent, several mice were inoculated with the medium alone. No reaction occurred during 28 days of observation.

Treatment with sulfanilamide and a derivative, sodium formaldehyde sulfoxylate-sulfanilamide.—The literature reveals that several investigators attempted experimental therapy with sulfanilamide in mice infected with streptococci falling under Group B by Lancefield's precipitation test. Only one group of investigators[5] indicated that *Str. agalactiae* was used as the infecting organism.

From their observations, Dawson and Hobby[6] concluded that sulfanilamide was

Contribution from the Dermatological Research Laboratory, division of Abbott Laboratories, Philadelphia.
Drs. Louis A. Klein and Albert L. Kleckner of the University of Pennsylvania supplied the culture and subsequently reidentified it.

[1]Minett, F. C., Stableforth, A. W., and Edwards, S. J.: Studies on Bovine Mastitis. J. Comp. Path. Therap., xlv (1932), pp. 1-10.
[2]Minett, F. C.: The Bacteriology of Bovine Streptococcus Mastitis. J. Hygiene, xxxv (1935), pp. 504-511.
[3]Minett, F. C., Stableforth, A. W., and Edwards, S. J.: Studies on Bovine Mastitis. J. Comp. Path. Therap., xlii (1929), pp. 213-230.
[4]Long, Perrin H.: Personal communication. Johns Hopkins Univ., Baltimore, Md.
[5]Long, Perrin H., and Bliss, Eleanor A.: The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds. The Macmillan Co., New York, (1939), p. 23.
[6]Dawson, M. H., and Hobby, G. L.: Observations on the Chemotherapeutic Effect of Sulfanilamide in Experimental Hemolytic Streptococcal Infections. J. Clin. Invest., xvii (1938), p. 521.

effective against experimental hemolytic streptococcal infection with Group B (Lancefield) organisms as well as non-hemolytic strains within Group B. No experimental data were included in the report. Buttle, Gray and Stephenson[7] reported that Colebrook obtained successful results with sulfanilamide in mice against one strain of streptococcus belonging to Lancefield's Group B. Bliss and Long[5] found that experimental infections in mice produced by Lancefield's Group B (bovine and human) strains of hemolytic streptococci were quite resistant to sulfanilamide. Feinstone[8] reported little success in the control of experimental Lancefield's Group B streptococcal infections in mice.

In several experiments in which we inoculated a total of 50 mice with lethal doses of the neopeptone blood broth culture, failure invariably resulted with sulfanilamide and sodium formaldehyde sulfoxylate-sulfanilamide. In these experiments, one group of mice was inoculated intraperitoneally with 0.5 cc. of undiluted culture which contained approximately 70 million organisms, and another group received approximately 14 million organisms. The drugs were given by gavage immediately after infection and three times daily thereafter at six-hour intervals. The sulfanilamide was suspended in 10 per cent gum acacia solution to insure a homogeneous mixture. Sodium formaldehyde sulfoxylate-sulfanilamide was given in aqueous solution. Ten milligram doses were administered. Nearly all of the animals died on the first day. Only one untreated control survived for four days. Doubling the dose of the drugs failed to exert any appreciable therapeutic effect.

Bliss and Long[9] and others,[7 10 11] in working with beta hemolytic streptococcal (Lancefield's Group A) infection in mice, noted a marked interference with the therapeutic action of sulfonamides when large broth inoculums of low virulence organisms were necessary for lethal doses. With the possibility in mind that the metabolic products of the organism present in the crude culture may partially interfere with drug therapy, physiological saline suspensions made from plated organisms were used to infect the mice.

In a preliminary experiment in which 30 mice were used, 1 cc. of saline suspension of *Str. agalactiae* containing approximately 420 million organisms was used as the lethal dose. All of the treated mice, as well as the controls, died within two days after infection. Upon reducing the number of organisms used for infecting the mice, radically different therapeutic results were obtained (see tables 1 and 2).

The tables are compilations of three separate experiments. The procedure was the same as previously outlined, except that the heart's blood of the last mouse, after passage of the organism, was streaked on blood agar instead of cultured in neopeptone blood broth.

The hearts of 20 mice that succumbed were cultured. The organism was recovered, mostly in pure culture, from 19. This number included mice dying as late as 8 to 10 days after infection or, in the case of treated mice, 3 to 5 days after treatment with the drugs was discontinued.

DISCUSSION

The use of physiological saline suspensions of *Str. agalactiae* for infecting mice revealed that sulfanilamide and sodium formaldehyde sulfoxylate - sulfanilamide had a therapeutic action. It is noteworthy that whereas these two drugs seemingly had no effect on mice infected with neopeptone blood broth cultures containing ap-

[7]Buttle, G. A. H., Gray, W. H., and Stephenson, Dora: Protection of Mice Against Streptococcal and other Infections by p - Amino - benzenesulfonamide and Related Substances. Lancet, ccxxx (1936), pp. 1286-1290.

[8]Feinstone, W. H.: Personal communication to Long, Perrin H., and Bliss, Eleanor A.: The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds. The Macmillan Co., New York, (1939), p. 217.

[9]Long, Perrin H., and Bliss, Eleanor A.: The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds. The Macmillan Co., New York, (1939), p. 17.

[10]Bloch-Michel, H., Conte, M., and Durel, P.: L'Emploi des Dérivés Sulfamidés non Azoique dans le Traitement de l'Érysipèle. Presse Méd., xliv (1936), pp. 1583-1584.

[11]Nitti, G., and Bovet, M.: Action du 4-Sulfonamide-2-4-Diaminobenzol (Prontosil) sur des Infections Streptococciques de la Souris Provoquées par des Streptocoques d'Origine Humaine. Compt. Rend. Soc. de Biol., clx (1935), pp. 1277-1280.

TABLE I.—Inoculums Undiluted. 0.5 cc. of Saline Suspension Inoculated Intraperitoneally Contained Approximately 225-255 Million Organisms.

	No. of Mice	% Survived 28 Days	% Succumbed Within Days						
			1	3	5	7	10	15	28
Controls	15	0	73	87	87	93	100	100	100
Sulfanilamide	15	13	27	40	40	67	80	87	87
Sodium formaldehyde sulfoxylate-sulfanilamide	10	0	20	30	40	60	90	100	100

Note the sharp rise in deaths of treated mice after the fifth day, at which time treatment was stopped.

TABLE II.—Inoculums Diluted One-half. 0.5 cc. of Saline Suspension Inoculated Intraperitoneally Contained Approximately 110-130 Million Organisms.

	No. of Mice	% Survived 28 Days	% Succumbed Within Days						
			1	3	5	7	10	15	28
Controls	15	13	53	73	80	80	87	87	87
Sulfanilamide	15	67	7	27	27	33	33	33	33
Sodium formaldehyde sulfoxylate-sulfanilamide	10	60	10	10	10	20	30	40	40

proximately 14 million organisms, they exerted a therapeutic action when an inoculum containing as many as 255 million organisms in saline suspension was used. Therapeutic action exerted by the drugs was even more marked when the infecting dose of organisms in saline suspension was halved.

CONCLUSIONS

Streptococcus agalactiae, which is ordinarily nonpathogenic in mice, assumes marked pathogenicity when passed through a series of mice.

The therapeutic action of sulfanilamide and sodium formaldehyde sulfoxylate-sulfanilamide seems to be inhibited in mice infected with *Str. agalactiae* in neopeptone blood broth medium.

Sulfanilamide and sodium formaldehyde sulfoxylate-sulfanilamide exert a specific therapeutic action in mice infected with *Str. agalactiae* in a saline suspension.

Experiments on the Life History of the Cattle Lungworm, Dictyocaulus Viviparus

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THE CATTLE LUNGWORM, *Dictyocaulus viviparus*, has long been recognized as the cause of verminous pneumonia and bronchitis in cattle. Because of the potential and, in many cases, actual damage produced by the cattle lungworm, it is desirable that pertinent facts concerning the course of infection and symptomatology of this parasitism be determined. The life history and host-parasite relationships of a related lungworm in sheep, *Dictyocaulus filaria*, have been quite thoroughly investigated (Romanovich and Slavine,[1] Guberlet,[2] Daubney,[3] Hobmaier and Hobmaier,[4] Kauzal,[5, 6] Shaw,[7] and others) but available information on the cattle lungworm is rather sketchy, except for studies by Daubney[3] on the development of the preparasitic stages. In view of the similarity in the development of the preparasitic stages of the sheep and cattle lungworms, it has been assumed generally that the lungworm of cattle, like its close relative that parasitizes sheep, has a direct life history. The facts that support that assumption are presented in this paper.

From the Regional Animal Disease Research Laboratory, U. S. Bureau of Animal Industry.

[1]Romanovitch, and Slavine, A.: Étude sur l'évolution du *Dictyocaulus filaria* (*Strongylus filaria*) et l'Inféstation des Moutons. Compt. Rend. Soc. Biol., Paris, lxxvii (1914), pp. 444-445.

[2]Guberlet, J. E.: On the Life History of the Lungworm, *Dictyocaulus filaria*, in Sheep (Preliminary Report). J.A.V.M.A., lv (1919), pp. 621-627.

[3]Daubney, R.: The Life Histories of *Dictyocaulus filaria* (Rud.) and *Dictyocaulus viviparus* (Bloch). J. Comp. Path. Therap., xxxiii (1920), pp. 225-264.

[4]Hobmaier, A., and Hobmaier, M.: Die Entwicklung des Lungenwurms des Schafes, *Dictyocaulus filaria*, Rud., ausserhalb and innerhalb des Tierkorpers. Münch. Tier. Wchnschr., lxxx (1933), pp. 20-26.

[5]Kauzal, G.: Observations on the Bionomics of *Dictyocaulus filaria*. Austral. Vet. J., ix (1933), pp. 20-26.

[6]Kauzal, G.: Observations on the Development of Resistance to *Dictyocaulus filaria*. Austral. Vet. J., x (1934), pp. 101-111.

[7]Shaw, J. N.: Lungworms (*Dictyocaulus filaria*, Rudolphi) in Sheep and Goats. Ore. Agr. Exp. Sta. Bul. 327, (1934), 12 pp.

Materials and Methods

With the exception of one mature cow that was used in one test, grade Jersey calves, purchased at birth from local dairies, were used in the experiments. The calves were kept in barns, the stalls of which were partitioned with wood and floored with concrete. The manure was removed and the floors cleaned with water daily to prevent extraneous parasitism. The calves were fed milk until about 5 months of age. Alfalfa hay and grain were put before them when they were about a week old and continued as their regular diet after weaning. Naturally infected cattle from nearby farms were used as a source of larvae for the infection experiments. First-stage lungworm larvae were isolated from freshly passed feces of infected cattle by means of a modified Baermann apparatus similar to that used by Shaw[7] in his studies on the sheep lungworm. Feces containing the larvae were suspended in fine gauze at the top of an 8-inch funnel which was filled with warm water. The tip of the funnel was attached to a conical test tube in which the larvae collected as they migrated from and settled through the feces. The larvae thus collected were placed on moist filter paper in small stender dishes and kept at room temperature (22 to 28 C.). When cultures were prepared in this manner, development of the larvae to the third or infective stage was completed in 5 to 7 days. Lungworm eggs are rarely eliminated as such, but hatch before they are expelled with the droppings. In our experience, eggs were seen in freshly passed feces on only one occasion. The parasitized animal was a calf that had a severe diarrhea associated with coccidiosis.

For experimental infections, infective larvae were washed from the filter paper and the number was estimated by dilution counting before they were administered. To inoculate a test animal, the larvae were drenched into the back of the mouth by means of a dissecting blowpipe attached to a 5 cc. rubber bulb. To follow the course of the infections, fecal examinations were begun about 15 days after inoculation. Examinations were made daily until appearance of larvae in the droppings and were continued at frequent intervals until larvae were no longer present in the feces. Large numbers of larvae were counted by the dilution technique and small numbers by the

flotation technique as described by Stoll.[8, 9] Because the first-stage larvae were killed immediately in the concentrated solution of sodium chloride that was employed in the flotation technique and were, therefore, difficult to detect, it was necessary to employ some other levitating medium. It was found that the larvae could be floated efficiently and would remain viable for at least half an hour in a solution of zinc sulfate having a specific gravity of 1.18. This solution, as used by Faust and his coworkers[10] for the diagnosis of protozoan cysts and helminth eggs, is made by dissolving 331.4 Gm. of $ZnSO_4.7\ H_2O$ in sufficient water to make 1.0 liter of solution. As the total number of larvae at the beginning and end of the patent period was usually quite small, usually the flotation technique was supplemented with the Baermann technique previously described. Negative flotations were checked by examining about 200 Gm. of fresh feces by the latter technique.

One calf died of an injury and two others were destroyed during the experiments. The remaining animals were subsequently used for other experimental work at the laboratory following recovery.

OBSERVATIONS AND DATA

The Course of Infection Following First Inoculations.—The results of feeding infective lungworm larvae to calves are summarized in table 1. As shown by the data presented in the table, larvae of *D. viviparus* appeared in the feces of the majority of the animals in from 24 to 30 days after inoculation. This prepatent period was as short as 21 days in calf 33, following the first inoculation, and as long as 41 days following reinoculation of calf 50, to be discussed later. No attempt was made to trace the migration of the larvae within the host, nor to determine the exact time when they reached the lungs. However, young female worms, 2.2 to 4.8 mm. long, were found in the lungs of calf 51 which was examined 13 days after larvae were fed. In view of the fact that mature females are usually 60 to 80 mm. long, the growth of these parasites must be very rapid after they reach the lungs.

The maximum number of larvae was observed in the feces during the fifth to seventh week after the prepatent period. The shortest interval between the date of appearance of larvae in the feces of the experimentally infected host and the date of maximum larvae production was observed during the infection of the older animals (calves 32, 52, and 87), which eliminated small numbers of larvae and in which the parasites were apparently short lived. The duration of infection, measured from the time of inoculation until larvae of the next generation were no longer present in the feces, was longer in younger than in older animals. The length of life varied from slightly over a month, in the case of the calves 208 to 342 days old, up to 4 months in calf 47, first infected when 6 days old. Postmortem examination of calf 97, killed 13 days after disappearance of larvae from the feces, failed to reveal lungworms. This finding indicates that the disappearance of larvae was coincident with the death of the worms in the lungs and their elimination from the host shortly thereafter.

Reinfection.—Attempts to reinfect the host following the loss of worms in the initial infection were made in the case of calves 33, 47, and 50 (table 1). Calf 33, first inoculated on Oct. 3, 1939, was fed 1,300 larvae on April 24 and 2,250 larvae on May 2, 1940, 126 and 134 days, respectively, after the termination of the first infection as determined by the absence of larvae in the feces. This animal subsequently did not pass lungworm larvae following these inoculations nor show any other evidence of infection. Calf 32, of the same age, was fed the same number of larvae from the same culture, on the same days calf 33 was reinoculated. Calf 32 did harbor a small number of adult lungworms, as shown by the presence of small numbers of larvae in feces passed between the twenty-sixth and thirty-second days after the first inoculation (table 1).

Calf 47 was first fed 200 infective larvae on Dec. 22, 1939, and reinoculated with

[8]Stoll, N. R.: On Methods of Counting Nematode Ova in Sheep Dung. Parasitol., xxii (1930), pp. 116-136.

[9]Stoll, N. R.: Observations on Cattle Nematode Infections with a Demonstration of Their Secondary Transmission to Grazing Sheep. J. Parasitol., xxii (1936), pp. 386-407.

[10]Faust, E. C., *et al.*: A Critical Study of Clinical Laboratory Technics for the Diagnosis of Protozoan Cysts and Helminth Eggs in Feces. Am. J. Trop. Med., xviii (1938), pp. 169-183.

TABLE I.—Data on Experimental Infections with Dictyocaulus viviparus Produced in Calves by Feeding Infective Larvae

CALF NUMBER	AGE WHEN INOCULATED	LARVAE FED (ESTIMATED)		INTERVAL BETWEEN INOCULATION AND APPEARANCE OF LARVAE IN FECES	MAXIMUM LARVAE PER GM. OF FECES	INTERVAL BETWEEN INOCULATION AND MAXIMUM NUMBER OF LARVAE IN FECES	INTERVAL BETWEEN INOCULATION AND DISAPPEARANCE OF LARVAE FROM FECES
	DAYS	DATE	NUMBER	DAYS	NUMBER	DAYS	DAYS
33	138	10- 3-39	6500	21	800	44	78
33[1]	342	4-24-40	1300	..	0	..	..
		5- 2-40	2250				
32	342	5- 2-40	2250	26	12	29	32[2]
47	6	12-22-39	200	26	60	41	120
47[1]	138	5- 2-40	6500	26	86	37	56
50	52	3-20-40	575	24	170[4]	48	87–90[3]
..	..	4-29-40	4250				
50[1]	344	1- 6-41	2100	41	2	44	50
51	91	4-29-40	4250[5]				
52	331	1- 6-41	2100	30	2	33	34
87	208	4-14-41	6000	30	2	33	35
96	26	10-26-40	1500	30	68	46	79
97	89	12-28-40	7500	24	70	32	54
98	151	2-28-41	1000	25	136	48	..[6]
141	13	5-20-41	2000	28	38	37	58

[1]Reinfection.
[2]24 days since second inoculation.
[3]50 days since second inoculation.
[4]194 larvae per Gm. of feces observed 24 and 26 days after second inoculation (see fig. 1).
[5]Died of injury 13 days after inoculation. Immature lungworms recovered.
[6]Killed 63 days after inoculation. 5 male and 2 female lungworms recovered. 8 larvae per Gm. of feces.

6,500 larvae on May 2, 1940, 132 days later. Larvae from the first infection had disappeared from the feces 12 days previously. It is quite possible that a larger number of worms was established from the second inoculation than from the first, as indicated by the larvae counts. A maximum of 86 larvae per Gm. of feces was observed 37 days after the second dose of larvae was fed. Although this was only 26 more than the largest number passed during the course of the first infection, the total number of larvae passed per day at this time was approximately 10 times greater, due to the fact that the calf at this age was passing seven times the amount of feces passed per day during the course of the first infection. The data on larvae counts after inoculation of calf 50 are shown graphically in figure 1. As shown by the figure, larvae were first detected in the feces of this animal 24 days after the initial inoculation on March 20, 1940. Forty days after the first inoculation, this calf was fed 4,250 additional larvae.

That additional worms were established is indicated by the recovery of 194 larvae per Gm. of feces 24 and 26 days after the second inoculation. This last peak was followed by a precipitous decrease in the number of larvae until they were no longer present 90 days after the first and 50 days after the second inoculation. This animal was still negative when fed 2,100 infective lungworm larvae 102 days later (figure 1). Larvae from the female worms in this inoculation were first detected on the forty-first day by using the modified Baermann technique. Two larvae per Gm. of feces were present on the forty-fourth day, after which the number of larvae became so small as to be detected only by Baermann isolation. Larvae disappeared entirely 50 days after reinoculation, or 241 days after experimental infection. Calf 52 served as control in this test. This calf, previously unparasitized,

was similarly inoculated at the same time that calf 50 was reinoculated. Small numbers of larvae were detected in the feces from the thirtieth to the thirty-third day. Aside from earlier maturity of the worms, there was no evidence that this calf was more susceptible to infection than was calf 50, which had a previous history of parasitism with lungworms.

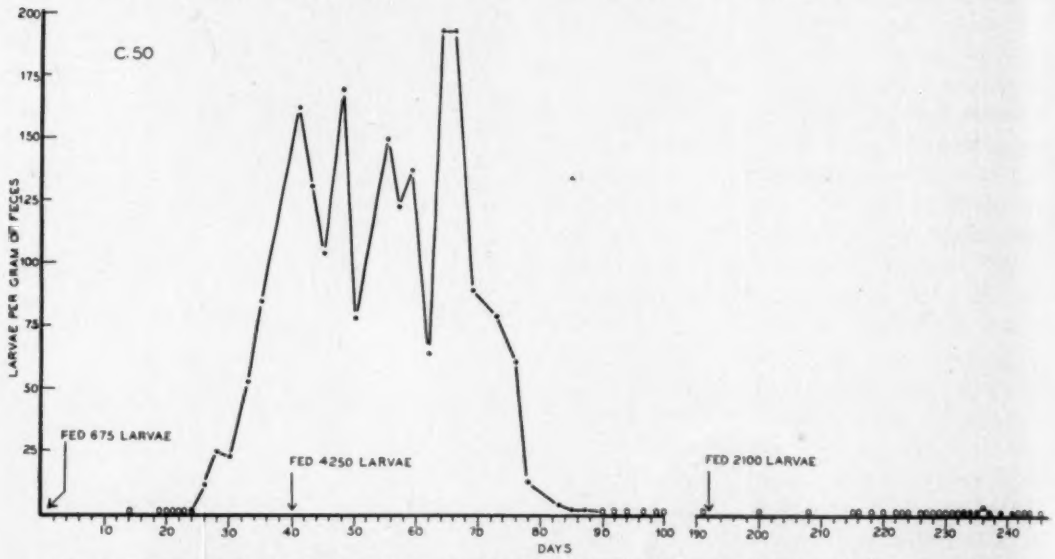

Fig. 1. Larvae counts obtained on feces of calf 50 fed infective lungworm larvae as indicated. Points on base line indicate positive samples, open circles indicate negative findings.

Effects of Lungworms on the Host.—Observations on the physical condition and activity of the hosts during the course of infection disclosed manifestations of pulmonary involvement and retardation of growth in the younger calves (33, 47, 50, 96, 97, 98), where it was evident from the larvae counts that a sizeable number of adult worms had become established in the lungs. Such symptoms as shallow, labored breathing, persistent coughing, and diminished appetite became evident about the time larvae appeared in the feces. Gradual symptomatic improvement followed elimination of the parasites. Calf 33 had an intermittent diarrhea between the thirty-fifth and fifty-first day of initial infection. Diarrhea was not observed in calf 32, serving at the time as an uninfected control. Diarrhea was not observed in any of the other animals during the course of experimental infection; however, the latter were not as heavily parasitized as was calf 33, judging from the number of larvae passed. Examination of the blood of calves 33, 50, 96, and 97 at weekly intervals during the course of infection failed to reveal any evidence of anemia or significant change in the number of leucocytes. A slower growth rate as a result of the infection was demonstrable by comparing the growth of infected and uninfected animals of the same age, kept under identical conditions on a similar diet. For example, calves 32 and 33 each weighed 135 lb. when the latter was first fed lungworm larvae Oct. 3, 1939. Calf 33 gained an average of 4.5 lb. per week between the date of inoculation and December 22, a week after larvae disappeared. Calf 32, the uninfected control of the same age, gained an average of 7.5 lb. per week during the same period. At the time calf 33 was reinoculated (Apr. 24, 1940), it weighed 370 lb., whereas calf 32 weighed 425 lb.

A further opportunity to observe the effects of this parasite on the growth of the host was afforded by recording the weights of calves 96, 97, and 98. These calves were inoculated in order, the first on Oct. 26, 1940, the second on Dec. 28, 1940, and the third on Feb. 28, 1941 (table 1). In this

way, calves 97 and 98 served as controls for calf 96, until the former was inoculated. Calf 98 then served as control for calf 97. Calf 96 gained an average of 5.1 lb. per week between the date of inoculation and December 28, a period of approximately 2 months, whereas calves 97 and 98 gained 7.2 and 6.3 lb. per week, respectively. Calf 97 gained an average of 10 lb. per week and calf 98 gained 12.2 lb. per week between December 28, when the former was inoculated, and February 28, the date the latter was fed infective larvae. In the period between October 26, when calf 96 was inoculated, and February 28, a period of 125 days, this animal gained a total of 128 lb., whereas calf 98, as yet uninfected, gained a total of 155 lb.

Prenatal Infection.—The possibility of prenatal infection of sheep and cattle with their respective species of Dictyocaulus has been recognized for some time. Neveu-Lemaire[11] reported finding adult *D. filaria* in the bronchi of a fetus *in utero* and also in a 4-day-old lamb. Kasparek[12] found calves only 1½ to 8 days old to be affected with lungworms. These findings, in the light of our present knowledge regarding the time necessary for development of the sheep and cattle lungworms, indicate intrauterine infection. One experiment conducted by the writers, however, suggests that prenatal infection in cattle is not readily established. In this experiment, a multiparous cow was fed a total of 30,600 infective larvae of *D. viviparus* over a period of one month, ending two weeks before parturition. The larvae were fed in doses of 2,100 to 7,000 at intervals of from 2 to 11 days. Frequent coughing on the part of the host was observed during the third to the seventh week after the first dose of larvae was given. However, lungworm larvae were not observed in its feces at any time although daily examinations were made over a period of two and one-half months. The calf born to this cow did not show any symptoms of lungworm disease, nor were larvae detected in its feces during daily examinations for the first two months of life.

[11]Neveu-Lemaire, M.: Les Premiers Stades Évolutif du Strongle Filaire. Bul. Soc. Zoöl. France, xxxvii (1912), pp. 238-241.

[12]Kasparek, Th.: Beitrag zur Prophylaxis der Lungewurmseuche. Arch. Tier., Berl., xxvi (1900), pp. 70-73.

DISCUSSION

The experimental infections described in this paper show that the development of the cattle lungworm is essentially similar to that reported for the sheep lungworm. According to Kauzal,[6] the prepatent period of the latter parasite was 25 to 35 days in 6-week-old lambs fed a single dose of larvae, and 37 to 41 days in 4- to 6-week-old lambs fed repeated doses of larvae. The former group threw off the infection in 66 to 103 days, and the latter group ceased to pass larvae in 64 to 71 days after the first dose of larvae was fed. Other investigators[2, 3, 7] found the interval between date of inoculation and appearance of larvae in the feces of lambs to range from 38 to 42 days. None of the animals in Shaw's[7] experiments remained infected longer than 56 days. Kauzal[6] and Shaw[7] found older sheep more resistant to experimental infection than younger ones, and the former presented evidence indicating that the age factor is more important in the host's resistance than is previous infection. According to the data presented in this paper, the prepatent period varied from 21 to 30 days following the first inoculation and the infection was thrown off in 32 to 120 days. Marked reinfection was obtained in one calf reinoculated when 138 days old. Reinfection failed in one short yearling; in another the worms matured slowly and lived only a short time. Although the results obtained in these short yearlings indicated an acquired resistance to reinfection, the initial infections in other short yearlings were of a mild type and of short duration, as compared with the longer, more severe infections established in the younger calves. The writers recently observed a herd of long yearling steers that had been continually exposed to reinfection on pasture. The majority of the animals in the herd were still infected with lungworms eight months after this parasitism was first diagnosed. An explanation of this fact is difficult in the light of the experi-

mental work described in this paper. It is possible that the life span is no longer under natural than under experimental conditions, but rather that some factors as yet undisclosed are responsible for reinfection occurring more readily in older animals under conditions of natural exposure.

SUMMARY

1) Experimental infections of cattle with the lungworm, *Dictyocaulus viviparus*, were established in calves by feeding the infective larvae. An attempt to infect a calf prenatally by feeding infective larvae to the pregnant dam gave negative results.

2) Zinc sulfate solution (sp. gr. 1.18) with Stoll's[8, 9] technique was employed to detect larvae in the feces and thus follow the course of infection.

3) Larvae appeared in the feces in from 21 to 30 days after inoculation; the prepatent period in a yearling previously parasitized was 41 days.

4) The duration of infection, measured from the time of inoculation until larvae of the next generation were no longer being passed in the feces, varied from about one month, in animals ranging from 208 to 342 days old at the time of inoculation, to four months in one calf inoculated when only 6 days old.

5) Reinfection was successful in a 4½-month-old calf, but only a slight infection was established in one of two short yearlings that were inoculated after worms from a previous infection had been naturally eliminated.

6) No symptoms were observed in primary infection and in reinfection of the older animals. Symptoms such as coughing, difficult breathing, and diminished appetite were seen in the more severe cases following initial inoculation in the younger calves. Intermittent diarrhea was observed in one calf having the most severe infection with lungworms. The gains in weight of infected animals were less than those of uninfected controls. There was no evidence of anemia nor significant change in the number of leucocytes in the blood of animals examined during the course of infection.

Analyses of Gas and Ingesta of Bloated Cattle

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EXPERIMENTAL STUDIES[1, 2] have shown that two toxic gases (carbon monoxide and hydrogen sulfide) common to the rumen are readily absorbed from the digestive tract, and that an increase in pressure in the rumino-reticular sac increases the rate at which these gases reach the blood stream.

It has also been shown[2] that concentrations of one of these gases (H_2S) can be increased, *in vivo*, by feeding good alfalfa hay and by placing cystine or inorganic sulfur in the rumen.

Relatively high intraruminal pressures have been maintained for several minutes in experimental cows with no apparent harmful effects.

It is felt, then, that there is a possible relationship between the termination of acute tympanites and the presence and amount of toxic gases in the rumen and other parts of the digestive tract. The advisability of correlating certain experimental evidence with findings in actual bloat cases has seemed highly desirable since this work began. The location of the station and certain geographic factors have made it difficult to carry on such studies either before or within a reasonable time after death.

Attempts to produce bloat by feeding methods have met with the same lack of success as has been experienced at other stations.

On Nov. 7, 1941, an opportunity was presented to make a study of material taken a few hours after death from three animals which had died of bloat. These animals were steers weighing over 800 pounds, in very good condition, having been in the feed lot three weeks. Prior to this time, they had been kept under better-than-average range conditions. Feeding and management conditions on this ranch are exceptionally good.

A mixture of first and second cutting chopped alfalfa hay was fed in these feed lots the first week. Grain feeding was started the second week and was gradually increased until each animal was getting seven pounds a day at the end of the third week. The grain ration consisted of one-half ground rye and one-half ground barley. A supplement had been added to the grain at the rate of one pound to eight pounds of grain.

PROCEDURE

Intraruminal Pressures in the Dead Animals.—Intraruminal pressures in the dead animals varied from 72 to 75 mm. of mercury. These pressures were approximately half as great as the maximum pressures which were established experimentally.[2]

The animals had been dead from three to twelve hours and had been kept in the shade. The prevailing temperature was quite cool at the time, so that it is felt that postmortem changes were kept at a minimum.

Gas samples were taken from the rumen of each animal. The ingesta was "frothy" in each of the animals. Sufficient liquid was expressed manually from the ingesta for chemical analyses. These samples were kept in airtight containers. One sample of abomasum ingesta was taken.

CHEMICAL ANALYSES OF INGESTA

		(Volumes % H_2S)
Gas Samples	No. 1	0.702
	No. 2	0.141
	No. 3	0.217
Ingesta Samples (Rumen)	No. 1	0.688
	No. 2	0.450
	No. 3	0.646
Ingesta Samples (Abomasum)	No. 3	1.208

Technical paper No. 400 of the Oregon Agricultural Experiment Station. From the Department of Veterinary Medicine, Oregon State College.

[1]Dougherty, R. W.: Physiological Studies of Induced and Natural Bloat in Dairy Cattle. J.A.V.M.A., xcvi (1940), pp. 43-46.

[2]Dougherty, R. W.: Further Investigations of Rumen Gases and Bloat in Ruminants. J.A.V.M.A., xcix (1941), pp. 110-114.

H_2S determinations were made using the method described by Christensen and Wong.[3]

Recent work on the paralyzing effect of histamine on the rumen suggested the advisability of making determinations of the histamine content of the ingesta. The histamine determinations were made spectrophotelometrically, using a modification of the extraction method described by Furman.[4] Final readings were made of histamine di-hydrochloride and, after conversion, recorded as histamine. (Histamine determinations were made by the Department of Chemistry.)

HISTAMINE CONTENT OF INGESTA

Rumen sample No. 1	0.966 mg. per 100 cc. of ingesta
Rumen sample No. 2	0.602 mg. per 100 cc. of ingesta
Rumen sample No. 3	2.454 mg. per 100 cc. of ingesta
Abomasum sample from steer 3	2.16 mg. per 100 cc. of ingesta

PROTEIN CONTENT OF FEED

Chopped alfalfa hay	12.11% protein
Grain—½ rye and ½ barley	11.64% protein
Concentrate (supplement)	25.62% protein

(Feed analyses were made by the Department of Agricultural Chemistry.)

DISCUSSION

In this type of work, it is an established fact that occasionally experimental data should be compared with that obtained from actual field cases. In submitting the data in this progress report, such an attempt has been made.

It is realized that data obtained from material collected from animals just before or soon after death should be more significant.

The relatively high concentration of H_2S in the material may be significant. The proof of its importance in field cases is yet to be determined. Rather extensive studies of the action of hydrogen sulfide have been carried on in coöperation with the department of chemistry. It was hoped that methods could be devised that would enable detection of the insidious manner in which the gas works. So far, such efforts have been fruitless. Small amounts of sulfhemoglobin formed in animals dying of acute H_2S poisoning are incidental and apparently have no bearing on the immediate cause of death. Briefly, there is at present no definite method of determining whether an animal has died of H_2S poisoning.

Work done at this station by Shaw, Muth, and Seghetti[5] and rumen motility experiments now in progress suggested that histamine might be a factor in bloat. From 2 to 5 mg. of histamine injected intravenously causes a complete paralysis of the rumen for 5 to 20 minutes. During this work, it has also been shown that paralysis of the rumen inhibits eructation.

It is an interesting fact that relatively large amounts of histamine were present in the ingesta samples.

The histamine content of rumen ingesta can be increased markedly by feeding protein-rich feeds. At the same time, the H_2S content also increases. The increase in histamine may be due to decarboxylation of histidine.

It is hoped that this brief report of data obtained from actual bloat cases may stimulate additional studies on actual cases when their proximity permits.

SUMMARY

Ingesta and gas samples were taken shortly after death from three steers that had died of acute tympanites.

All gas and ingesta samples had relatively high concentrations of H_2S.

All ingesta samples were relatively high in histamine content.

Existing pressures in the ruminoreticular cavities of the dead animals varied between 72 and 75 mm. of mercury.

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Parasites of Moose in Northern Minnesota

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THE HELMINTH and arthropod parasites recovered from moose (*Alces americana americana* (Clinton)) in northern Minnesota have been reported by Fenstermacher and Jellison,[1] Wallace,[2] Fenstermacher[3, 4, 5] and Jellison.[6] More recently, Dikmans[7] summarized the literature on the helminth parasites infesting North American semi-domesticated and wild ruminants, in which he listed all the forms known to infect the moose. In addition to the species reported by Wallace, Fenstermacher, and Jellison, Dikmans' list contained the fringed tapeworm (*Thysanosoma actinioides*) from a moose taken in the Yellowstone National Park, Wyoming.

Since the publication of the lists of parasites by these authors, 10 additional animals from Minnesota have been examined. Eight of these specimens were represented by entire carcasses, one of which was a 7-year-old bull that had been held in captivity at Itasca State Park since calfhood. The other two individuals were represented only by portions of the carcass. From these 10 animals, 12 species of parasites were obtained, 9 of which have been reported by the above mentioned authors. Three nematode parasites heretofore unreported for moose in North America were found to constitute new records. They are the whipworm (Trichuris sp.), 17 specimens; the nodular worm (*Oesophagostomum (H.) venulosum*), 22 specimens; and a large intestinal round worm (Ascaris sp.), 2 female specimens.[2] The first two species were found in the animal confined in Itasca Park. White-tailed deer (*Odocoileus virginianus*) roamed freely outside the moose enclosure, which also was near to the one containing the herd of bison (*Bison bison*). According to Dikmans,[7] the nodular worm has been reported from the white-tailed deer. All three species of parasites occur commonly in domestic ruminants.

In considering the species of whipworm involved, Dr. G. Dikmans, of the United States Bureau of Animal Industry, in a personal letter[8] stated that "The question of the species of Trichuris found in domestic and wild ruminants has not been sufficiently studied . . . (and) . . . The present status of this group is a constant annoyance." In deference to this opinion, it appears best to designate these nematodes as Trichuris sp. until further studies have been made on the group. Wetzel and Enigk[9] and Hinemann[10] reported the whipworm (*Trichuris globulosa*) and Hinemann[10] the nodular worm (*O. (H.) venulosum*) from the European elk (*Alces alces*).

Chapin[11] described as *Dictyocaulus hadweni* a nematode found in the lungs of the American bison at Wainwright, Alberta, Canada, which was reported later from the moose by Wallace.[2] Dikmans[12] found that the range of variation in the bursal rays and spicules of *Dictyocaulus viviparus* (Block, 1782) included those of *D. hadweni*. His conclusion that *D. hadweni* is a synonym of *D. viviparus* is concurred in by the writers.

The frequency with which the various parasites were found in the moose examined is shown in table I. The percentage of infections is based on the number of possibilities for the parasites to be found, depending upon whether the entire carcass or only a portion of it was available for examination.

The distribution of the species of parasites in the individual animals showed that

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multiple infections are the rule. Of the 15 species of parasites listed in table I, the six occurring most commonly are, in the order of frequency, the winter tick (*Dermacentor albipictus*); two species of nematodes, the small intestinal round worm (*Nematodirella longispiculata*) and the lung worm (*D. viviparus*); the bladder worm (*Cysticercus tenuicollis* = *Taenia hydatigena*); one species of larval cestode, the hydatid cyst of *Echinococcus granulosus;* and one species of fluke, the rumen fluke (*Paramphistomum cervi*). The remaining nine species occurred more rarely.

While the six most common species, judging from the frequency with which they occurred and their relative numbers in most cases, may be the causes of poor health in moose, death was diagnosed definitely in only one case as being caused by a parasite; namely, the large liver fluke (*Fascioloides magna*). Seyfarth[13] reported that calves of A. *alces,* infected with large numbers of the rumen fluke, became so emaciated as the result of widespread destruction of the stratum corneum of the rumen that they had to be killed. No evidence of such a lesion was observed in *A. americana.* The probable importance, however, of a larval nematode found in the brain stem of one animal (Fenstermacher[3, 4]) should not be overlooked, although its significance is not known definitely.

In most instances, the effect of the various parasites, individually and collectively, is difficult to evaluate in wild animals, where opportunities for their study are limited. It is felt, however, that a general discussion of the relative abundance of the parasites in the order of their frequency will be of value to persons interested in the diseases and parasites of moose.

ARTHROPODA

The only arthropod which occurred in any significant numbers was the winter tick (*D. albipictus*). It was found frequently in great numbers (Fenstermacher and Jellison[1]), occurring on 67 per cent of the animals examined, in every month of the year in which moose were collected except July. Of the animals reported as harboring ticks, seven were designated as being very heavily infested. The photographs published by Fenstermacher and Jellison[1] bear witness to the extent and seriousness of some of the infestations.

The only other arthropod reported is a single specimen of black fly (*Simulium venustum*) collected from a sick female moose killed at Grand Marais on Oct. 11, 1933. The relationship may have been purely coincidental.

Although but a single important species of arthropod parasite has been found infesting Minnesota moose, the European elk, considered by some authorities to be the same as the American moose, is known to harbor at least six species, including two mites and four flies. Mangy conditions appearing on *A. alces* in Germany were caused by Psoroptes and Sarcoptes mites. Psoroptes mange was observed to occur on emaciated animals, appearing as bare patches particularly on the cape at the base of the bell, in the mane, and on the skin folds of the shoulder. A fatal case of sarcoptic mange was observed in one young animal (Ullrich[14]). Two nasal botflies (*Cephenomyia ulrichii* and *Pharyngomyia picta*), the deer hippoboscid fly (*Lipoptera cervi*), and the deer warble (*Hypoderma diana*) were found in Germany by Eichler.[15] Nose bots were reported as the probable cause of death in one specimen of *A. alces* in Germany by Wetzel and Enigk.[16]

The occurrence of nasal bots in *A. alces* is of interest since they have never been reported from *A. americana,* although the nasal bot (*Cephenomyia phobifer*) is common in deer throughout northern Minnesota.

Two species of nematodes, *N. longispiculata* from the small intestine and *D. viviparus* from the lungs, occurred in 46.6 and 42.4 per cent of the 30 and 33 viscera, respectively, available for examination. Extensive intestinal enteritis was present in cases where infection with *N. longispiculata* was heavy.

Four other species of nematodes were found on rare occasions; namely, the body-cavity filariid (*Setaria cervi*), the nodular

worm (*O.* (*H.*) *venulosum*), the whipworm (Trichuris sp.) and a large intestinal worm (Ascaris sp.). The three latter species constitute new records for *A. americana.*

In addition to the five species of nematodes reported above, two specimens of interest have been found. Fenstermacher[3, 4] found an immature nematode in the brain stem of one sick moose. It is of interest to note that a similar infection of the spinal cord of *A. alces* in Germany by an immature filariid was reported by Wetzel and Enigk.[17] An immature female nematode recovered by Fenstermacher[5] from the anterior chamber of the eye of an adult female moose was reported by Dikmans and Wehr[18] as probably belonging to the Elaphostrongylus.

The list of nematodes reported for *A. alces,* which includes 11 species, is considerably longer than that for *A. americana,* but no data are given in regard to their incidence. For the convenience of workers who have the opportunity to make parasite studies or collections from the moose, the list of nematodes reported for *A. alces* is included as an aid for the interpretation of their findings. Species marked with an asterisk have been found also in *A. americana.*

SPECIES	COMMON NAME	HABITAT	AUTHORITY
**Dictyocaulus viviparus*	Large lung worm	Lung	Wetzel and Enigk[9]
Haemonchus contortus	Large stomach worm	Abomasum	Wetzel and Enigk[19]
Ostertagia circumcincta	Small stomach worm	Abomasum	Wetzel and Enigk[17]
Muellerius capillaris	Small lung worm	Lung	Wetzel and Enigk[17]
Filariid	Immature filariid	Spinal cord	Wetzel and Enigk[17]
Strongyloides papillosa		Small intestine	Wetzel and Enigk[9]
**Setaria cervi*	Filariid	Body cavity	Wetzel and Enigk[9]
**Oesophagostomum venulosum*	Nodular worm	Colon	Hinemann[10]
Bunostomum trigonocephalum	Hookworm	Small intestine	Hinemann[10]
Nematodirus filicollis		Small intestine	Hinemann[10]
Trichuris globulosa	Whipworm	Cecum	Hinemann[10]

CESTODA

Cestodes, particularly the larval stages, constitute one of the commonest parasitic infections encountered in *A. americana.* Two larval and two adult forms of tapeworms have been identified definitely. Wallace[2] reported Cysticercus from the cardiac muscles of two moose that resembled *Cysticercus cellulosae,* but a critical study of them was not made to ascertain their identity. Attempts to obtain adult forms by feeding Cysticercus found in the myocardium to a fox by Christensen (Fenstermacher[3]) and to dogs by Fredine (Fenstermacher and Jellison,[1] Fenstermacher[3]) and by the authors were unsuccessful. The Cysticercus found in the liver were shown by Wallace,[2] by Fredine (Fenstermacher and Jellison,[1] Fenstermacher[3]) and by the authors to develop into *T. hydatigena* in dogs in 63 days. In addition to Cysticercus in the heart, one moose harbored numerous cysts in the skeletal muscles and some in the intestinal mesentery. It is the authors' opinion that the Cysticercus found in the myocardium and mesentery, as well as those from the skeletal muscles of moose in northern Minnesota, are those of *T. hydatigena* which occur so commonly in the adult form in the coyotes and wolves of that region.

Feeding experiments conducted by Wetzel and Enigk,[16] in which they fed Cysticercus from the heart and masseter muscles and from the mesentery and liver to dogs, produced mature *T. hydatigena* in 63 and 66 days, respectively.

The hydatid cysts of the tapeworm (*E. granulosus*) were found commonly in the lungs and occasionally in the liver of *A. americana.* Wallace[2] and Fredine (Fenstermacher[5]) ascertained their identity by feeding the cysts to dogs from which they obtained large numbers of the adult worms. Hadwen[20] reported hydatid cysts from moose in Manitoba, and Law and Kennedy[21] found a heavy infection in the lungs of an Ontario moose.

The adults of *E. granulosus* occur com-

TABLE I.—The Occurrence of the Parasites of Minnesota Moose in the Order of their Frequency.

SPECIES OF PARASITES	COMMON NAME	LOCATION IN/ON BODY	NO. TIMES IT OCCURRED	NO. OF POSSIBILITIES BECAUSE OF NATURE OF MATERIAL EXAM.	PERCENTAGE OF MOOSE INFECTED*
Dermacentor albipictus	Winter tick	Skin	24	36	66.6
Nematodirella longispiculata		Small intestine	14	30	46.6
Dictyocaulus viviparus	Lungworm	Lungs	14	33	42.4
Cysticercus tenuicollis	Bladderworm	Mesentery, liver, cardiac and skeletal muscles	13	34	38.2
Echinococcus granulosus	Hydatid tapeworm	Lungs, liver	12	33	36.3
Paramphistomum cervi	Rumen fluke	Rumen	9	30	30.0
Moniezia benedeni	Tapeworm	Small intestine	5	31**	16.1
Fascioloides magna	Liver fluke	Liver	4	33	12.1
Setaria cervi	Body cavity filariid	Coelom, mesentery	4	30	13.3
Cysticercus sp.	Bladderworm	Liver	2	34	5.8
Oesophagostomum (H.) *venulosum*	Nodular worm	Colon	1	31	3.2
Trichuris sp.	Whipworm	Cecum	1	31	3.2
Ascaris sp.	Ascarid	Small intestine	1	31	3.2
Elaphostrongylus sp.		Anterior chamber of eye	1	34	2.9
Simulium venustum	Black fly	Skin	1	?	?

*While a total of 36 moose have been examined, they have not been represented in all cases by entire carcasses.

**One of these determinations was based on the presence of anoplocephalid ova in a fecal sample.

monly in the timber wolves inhabiting the moose range. Riley[22, 23, 24] reported that of 12 timber wolves (*Canis lupus lycaon*) examined, five harbored the adult worms. Coyotes were found not to be infected (Riley[23, 24]), but this is not to be considered as evidence that they do not act as a definitive host.

Two species of adult cestodes are reported for North American moose. The only one found in Minnesota is *Moniezia benedeni* which occurred in five animals, two of which were reported by Jellison.[6] It is doubtful that these are the cause of any unhealthy condition in the moose. The fringed tapeworm (*T. actinioides*) was reported by Dikmans[7] from a moose at Yellowstone National Park.

Moniezia denticulata was reported from *A. alces* in Germany by Hinemann.[10]

TREMATODA

Two species of flukes were found in *A. americana*. They are the large liver fluke (*F. magna*) and the rumen fluke (*P. cervi*). The rumen fluke is by far the commoner, but probably causes little or no damage to the adult host. At least one death has been attributed to the large liver fluke; more than 500 specimens were estimated to occur in a two-year-old male which was found sick in the woods but died during the night before being examined. In this instance, extensive damage to the liver was observed, and fluke ova were found distributed widely between the layers of the mesentery. Ordinarily the infections are light, and little if any serious damage is discernible.

Three additional species of liver flukes reported for *A. alces* have not been found, as yet, in *A. americana*. They are the common liver fluke (*Fasciola hepatica*) (Wetzel

and Enigk[17, 18]), the lanceolate fluke (*Dicrocoelium lanceolatum*) (Szidat,[25] Wetzel and Enigk[18]), and *Parafasciolopsis fasciolaemorpha* (Ejsmont,[26] Ullrich,[27] and Wetzel and Enigk[9]).

Key for field identification of the parasites known to occur in North American moose.*

(1) External parasites, ticks: *Dermacentor albipictus* (Packard) Internal parasites, worms 2

(2) Flat worms; flukes in liver or rumen and tapeworms, adults in intestine or larval forms, as bladder worms (Cysticercus), encysted in clear vesicles on mesentery, liver, heart and skeletal muscles, or as thick-walled hydatid cysts in lungs or liver..... 3
Round worms in body cavity, eyes, lungs, or intestine 8

(3) Flukes in liver or rumen..... 4
Tapeworms as adults in intestine, or bladder worms, or hydatid cysts..... 5

(4) Large, leaf-shaped flukes found in liver; liver flukes: *Fascioloides magna* (Bassi, 1875).
Small, rounded, reddish flukes found attached among villi of rumen; rumen flukes: *Paramphistomum cervi* (Schrank, 1790).

(5) Adults elongated, many segmented tapeworms in small intestine 6
Larval stages encysted on mesentery or liver, in skeletal or cardiac muscles or in lungs 7

(6) Adult worm with posterior margin of each segment smooth: *Moniezia benedeni* (Moniez, 1879).
Adult worm with posterior margin of each segment distinctly fringed: *Thysanosoma actinioides* (Diesing, 1835).

(7) Thin-walled clear vesicles (Cysticercus) on mesentery, liver, or in skeletal or cardiac muscles: *Cysticercus tenuicollis* = *Taenia hydatigena* (Pallas, 1766).
Thick-walled, opaque, hydatid cysts in lungs or liver (rarely): *Echinococcus granulosus* (Batsch, 1786).

(8) In eyes or body cavity, free or in mesentery of latter: *Setaria cervi* (Rud., 1819), and Elaphostrongylus (?) sp.
In lungs or intestinal tract..... 9

(9) In bronchi and bronchioles of lungs: long and thread-like lungworms: *Dictyocaulus viviparus* (Bloch, 1782).
In intestinal tract.....10

(10) Large worms, approaching a lead pencil in size, in small intestine: Ascaris sp.
Small worms, never remotely approaching the above size.....11

(11) Worms with slender, thread-like anterior ends and thicker posterior ends, in cecum; whipworm: Trichuris sp.
Worms without thread-like anterior end, bodies of same diameter throughout length 12

(12) Long slender worms, males 11-16 mm., females 13-24 mm., in large intestine; nodular worm: *Oesophagostomum* (*H.*) *venulosum* (Rud., 1809).
Long slender worms, males 20-25 mm., females 45-50 mm., in small intestine: *Nematodirella longispiculata* (York and Maplestone, 1926).

*The author explains the above tabulation as follows:

The figures on the left hand side of the page refer to the different types of parasites found in the moose. For example, (1) on the left hand side of the page, "external parasites, ticks," shows that the only ticks which have been found on moose are the *Dermacentor albipictus* Packard; therefore that is the end of the key.

The figures on the right hand side of the page refer to the numbers on the left hand side, *i. e.*, under (1) "internal parasites, worms," leads to the figure 2, which indicates that reference should be made to (2) on the left hand side of the page. Here will be found listed flatworms, flukes in the liver or rumen, tapeworms, etc. Any other parasites in that location not listed in the first paragraph will be found in 3 as indicated on the right side of the page; therefore, refer to (3) on the left hand side. If this does not apply to the parasite being identified, then the key on the right hand side refers only to figure 4. Refer to (4) on the left hand side and the final identification will be found of those flukes which were found in the moose. Since that is the final identification, it is the end of the key as far as that parasite is concerned.

Referring to (2) on the left hand side of the page—"roundworms in body cavity, eyes, lungs or intestines" leads to the figure 8 on the left side. To complete this identification, refer to 8 on the right side, and continue as indicated above.

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